THE SELECTIVE BACTERICIDAL ACTION OF STAINS CLOSELY ALLIED TO GENTIAN VIOLET.*

By JOHN W. CHURCHMAN, M.D.

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PLATES 62 TO 65.

The recent observation of a sharp selective bactericidal property possessed by gentian violet[1] raised the question as to which part of the gentian violet molecule was responsible for the sensitive affinity. It seemed wise to attack this problem by studying substances closely allied to gentian violet in chemical structure,[2] and the results obtained will be briefly reported in the present communication.

TECHNIQUE.

The technique employed was that described in the original paper.[3] The effect of the substance on the organisms studied was investigated both by applying it directly to the organisms themselves (vital staining) and by introducing it into the media on which they were planted. The method of transplantation on divided plates proved exceedingly valuable in this study. Aqueous solutions of the dyes were used, except in the case of rosanilin and pararosanilin, which are practically insoluble in water. Alcoholic solutions of these dyes were therefore employed. Inasmuch, however, as the reaction to be tested was a selective one it was not expected that the use of alcohol would interfere with the study, particularly as equally constant results had been obtained with gentian violet whether aqueous or alcoholic solutions were employed; and the findings justified this expectation.

The strength of dye used in the divided plate experiments was roughly estimated by the degree of staining of the agar. My experience with gentian violet had shown this method to be sufficiently accurate; and it will be seen in the photographs that the sharp selective effect of the dyes could be readily demonstrated in this crude quantitative way.

* Received for publication, December 20, 1912.

[1] Churchman, J. W., *Jour. Exper. Med.*, 1912, xvi, 221; Churchman, J. W., and Michael, W. Howard, *ibid.*, 822.

[2] I am indebted to President Remsen, of Johns Hopkins University, for advice on this point.

[3] Churchman, J. W., *loc cit.* It should be stated that in making the divided plates I now use thin zinc strips instead of the aneurysm metal first employed.

ORGANISMS STUDIED.

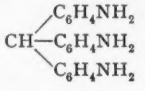
It was, of course, out of the question to study the effect of each dye on all the 334 strains employed in the investigation with gentian violet. Four type organisms were therefore selected. Two of these (*Bacillus anthracis* and *Micrococcus aureus*) had been found to be sharply violet-positive; the other two (*Bacillus coli communis* and *Bacillus typhosus*) had been found to be sharply violet-negative.[4] That is to say, when either of the first two organisms is stroked across a gentian violet divided plate no growth whatever occurs in the half of the plate containing the dye, whereas the last two organisms grow equally well on the two halves.

DYES STUDIED.

The following dyes were studied: dahlia, parafuchsin, magenta, pararosanilin, rosanilin, crystal violet, and methyl violet 5B.[5] Two series of transplants were made. In the first series the effect of the dye in question was studied by the method of vital staining. On a plain agar plate two strokes of *Bacillus anthracis* and two strokes of *Bacillus coli* were made. One stroke of each pair contained unstained organisms for control; the other stroke of each pair contained organisms stained with the dye that was being studied. The results are shown in figures 1 to 7. The controls, marked A and C, have grown luxuriantly. The stained *Bacillus anthracis,* marked A′, has constantly refused to grow, not even the track of the inoculating needle being visible in the photographs although this was to be seen in the plates themselves. On *Bacillus coli* the dye was without effect, the stained organisms, marked C′, grow-

[4] In my first communication, organisms, the growth of which was prevented by gentian violet, were referred to as violet-positive; those whose growth was uninfluenced by the dye were called violet-negative.

[5] A number of experiments were also done with paraleucanilin.



This dye is absolutely insoluble in water, and on adding alcoholic solutions of it to melted agar the dye is precipitated out. The results were on this account so unsatisfactory that they are not included in this report.

ing as luxuriantly as the controls, marked C. These plates were made after exposure of the organisms to the dye for two hours. The results of longer exposures (twenty-four and forty-eight hours) are shown in table I. They are identical with those obtained with gentian violet.

In the second series divided plates were used, the lower half of the plate containing plain agar, the upper half agar to which the dye in question had been added. These were stroked with *Bacillus anthracis, Micrococcus aureus, Bacillus coli,* and *Bacillus typhosus.* The results for the various dyes were constant, and are shown in figures 8 to 14. A comparison with figure 15 shows that the results with the allied dyes were identical with those obtained when gentian violet was used. The growth of *Bacillus coli* and *Bacillus typhosus* was uninfluenced by the dyes, that of *Bacillus anthracis* and *Micrococcus aureus* was entirely prevented.

One is not justified in making positive statements as to the effect of the dyes on organisms not actually studied; yet there is strong evidence for believing that an extension of the study to include the large number of organisms whose behavior was reported in the paper on gentian violet would parallel the findings for that dye. For it seems probable that these dyes, which have been found to have identical effects on four type organisms, owe this common property to a chemical group common to all of them; and that, therefore, they would have identical effects on any organism studied.

PARALLELISM WITH THE GRAM STAIN.

In my first paper attention was called to a parallelism existing between the Gram stain and the gentian violet reaction. About 90 per cent. of the Gram-positive organisms were found to be also violet-positive, and about the same percentage of Gram-negative organisms to be violet-negative. It seemed to me of some importance to see if a similar relation could be established in the case of the allied dyes; that is, whether the organisms whose growth was retarded by the dye would retain it when stained by the Gram method, the dye in question being substituted for gentian violet, and whether the organisms whose growth was uninfluenced would be

decolorized. These experiments were not wholly satisfactory as some of the dyes stained the organisms poorly. But I was unable to establish for the allied dyes such a relation as had been found to exist for gentian violet.

DIFFUSION OF THE DYE.

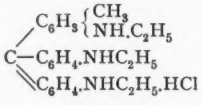
Attention was called in my first communication to the striking diffusion of gentian violet into the organisms on the plain agar side of a divided plate, the stain being frequently observed in colonies lying a centimeter or more from the dividing line. This phenomenon was also observed in the study of the allied dyes, an instance of it being shown in figure 16, which was painted from a parafuchsin divided plate. It will be seen that the entire *Micrococcus aureus* stroke is stained a deep pink at a distance of three centimeters from the dividing line, although the surrounding agar is not even tinted. This phenomenon suggests a strong affinity between organisms and stain.

CHEMICAL CONSIDERATIONS.

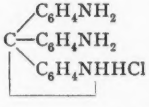
The dyes studied have the following chemical structure:

Gentian violet. A mixture of crystal violet, methyl violet, and dextrin.

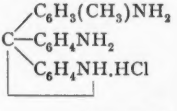
Dahlia.



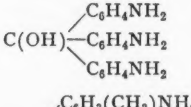
Parafuchsin.



Magenta (or fuchsin). A mixture of hydrochloric acid and acetic acid rosanilin, and pararosanilin.



Pararosanilin.

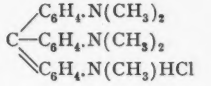


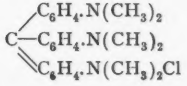
Rosanilin.

Crystal violet.

$$C\begin{cases} C_6H_4.N.(CH_3)_2 \\ C_6H_4.N.(CH_3)_2 \\ C_6H_4 \\ N.(CH_3)_2Cl \end{cases}$$

Methyl violet 5B (Merck). Double salt of penta- and hexamethylpararosanilin.





It will be seen that these dyes are closely related chemically. The determination of the chemical group responsible for their selective bactericidal power is the subject of investigations now under way in the laboratory of surgery at Yale University.

TABLE I.

Stain.	Organism.	Control.	Stained for 2 hrs.	Stained for 24 hrs.	Stained for 48 hrs.
Dahlia.....	B. coli communis	++	++	++	++
	B. anthracis	++	0	0	0
Parafuchsin.....	B. coli communis	++	++	++	++
	B. anthracis	++	0	0	0
Magenta.....	B. coli communis	++	++	++	++
	B. anthracis	++	Few scattered colonies	0	0
Pararosanilin (alcoholic solution)....	B. coli communis	++	++	++	++
	B. anthracis	++	0	0	0
Rosanilin (alcoholic solution).....	B. coli communis	++	++	++	++
	B. anthracis	++	0	0	0
Crystal violet.....	B. coli communis	++	++	++	++
	B. anthracis	++	0	0	0
Methyl violet 5B...	B. coli communis	++	++	++	++
	B. anthracis	++	0	0	0

EXPLANATION OF PLATES.

PLATE 62.

FIGS. 1 to 7 show the results of staining *B. anthracis* and *B. coli* with the dyes allied to gentian violet. The control strokes are marked A and C; the strokes of stained organisms A′ and C′.

FIG. 1. Dahlia.

FIG. 2. Parafuchsin.

FIG. 3. Magenta.

FIG. 4. Pararosanilin.

FIG. 5. Rosanilin.

PLATE 63.

FIG. 6. Crystal violet.

FIG. 7. Methyl violet.

FIGS. 8 to 15 show the results when *B. coli, B. anthracis, M. aureus,* and *B. typhosus* are transplanted on divided plates.

FIG. 8. Dahlia divided plate.

FIG. 9. Parafuchsin divided plate.

FIG. 10. Magenta divided plate.

PLATE 64.

FIG. 11. Pararosanilin divided plate.

FIG. 12. Rosanilin divided plate. (Stroked with *B. typhosus, B. anthracis, M. aureus,* and *B. coli,* from left to right in the order named.)

FIG. 13. Crystal violet divided plate.

FIG. 14. Methyl violet divided plate.

FIG. 15. Gentian violet divided plate.

PLATE 65.

FIG. 16 represents a parafuchsin divided plate stroked with *B. coli, B. anthracis, M. aureus,* and *B. typhosus,* from left to right in the order named. *B. coli* and *B. typhosus* grow equally well on the two sides of the plate, while *B. anthracis* and *M. aureus* refuse to grow in the upper half. The deep staining of the *M. aureus* stroke on the plain agar side is well shown. The track of the inoculating needle is seen in the upper half of the plate above the growths of *B. anthracis* and *M. aureus.*

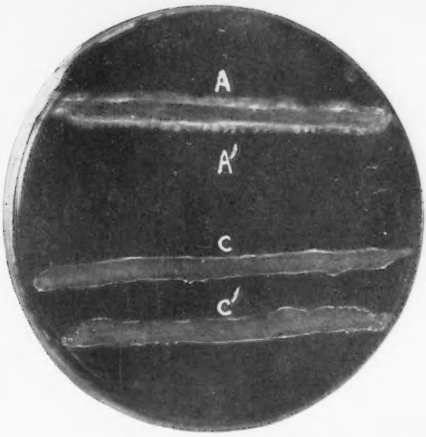


FIG. 1.

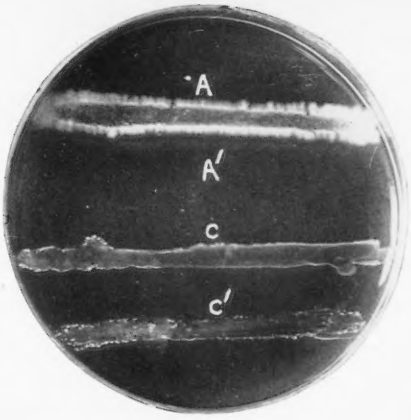


FIG. 2.

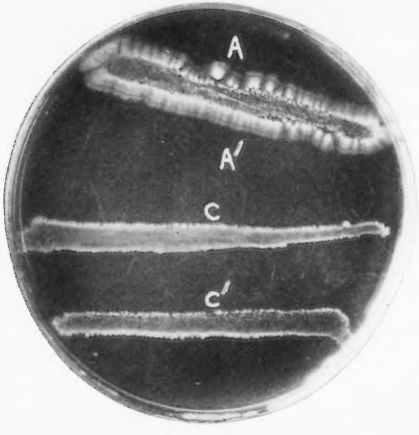


FIG. 3.

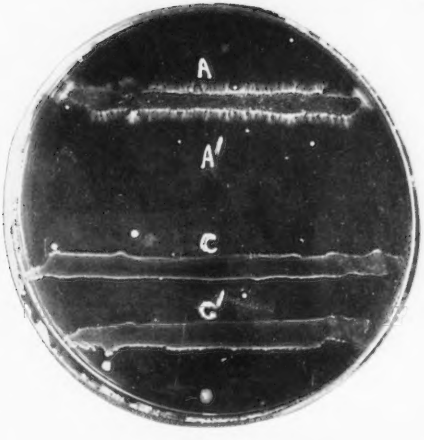


FIG. 4.

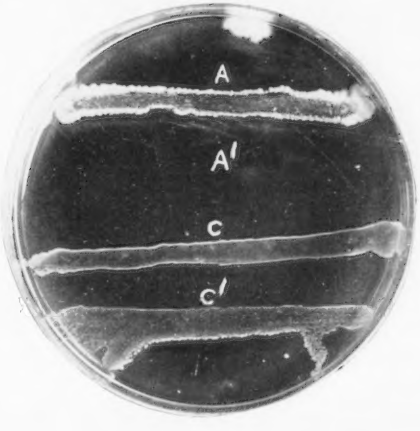
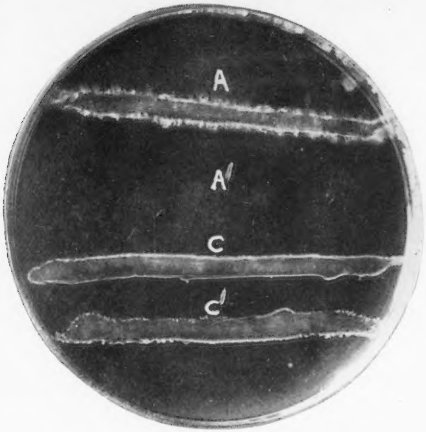


FIG. 5.

(Churchman : Selective Bactericidal Action of Stains.)



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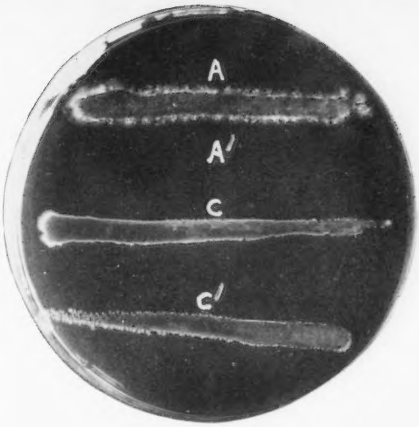

FIG. 7.

FIG. 8.

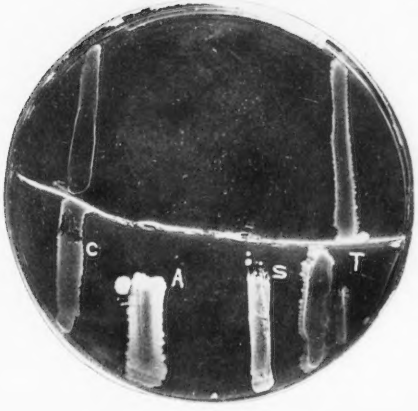

FIG. 9.

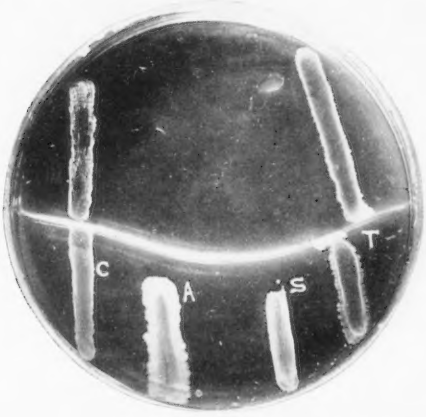

FIG. 10.

(Churchman : Selective Bactericidal Action of Stains.)

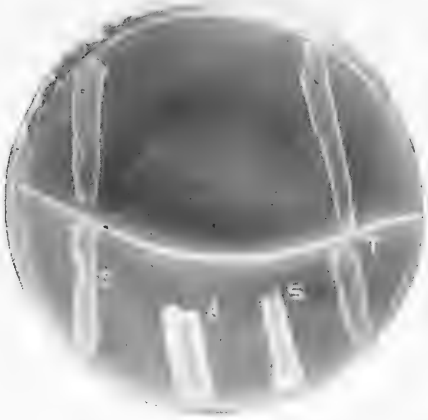

FIG. 11.

FIG. 12.

FIG. 13.

FIG. 14.

FIG. 15.

(Churchman: Selective Bactericidal Action of Stains.)

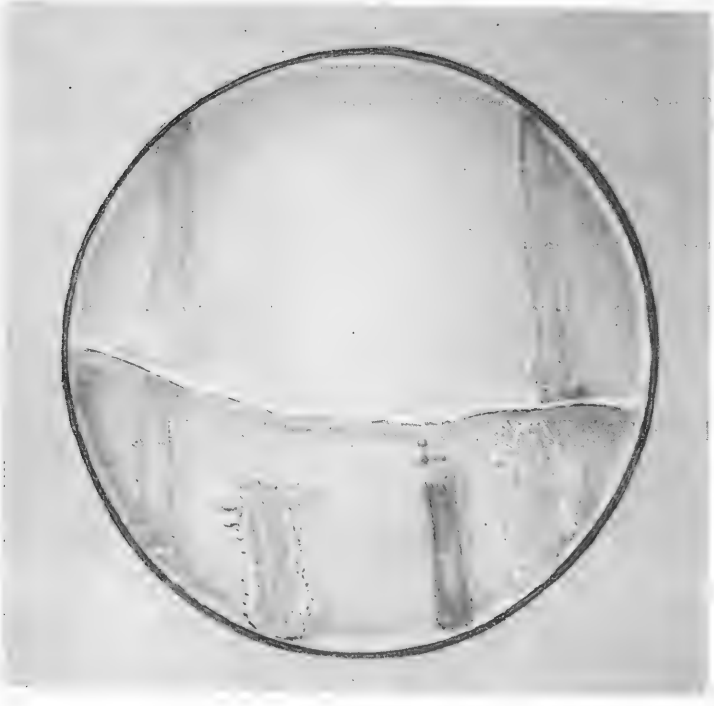

FIG. 16.

(Churchman : Selective Bactericidal Action of Stains).

THE METAMORPHOSIS OF THE ENDOSTYLE (THYROID GLAND) OF AMMOCOETES BRANCHIALIS (LARVAL LAND-LOCKED PETROMYZON MARINUS (JORDAN) OR PETROMYZON DORSATUS (WILDER)).*[1]

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Plates 66 to 70.

INTRODUCTION.

Wilhelm Müller in 1873 homologized the endostyle, or hypobranchial groove, of the Tunicate, Amphioxus, and the larval Petromyzon with the thyroid gland of all higher chordates. Subsequent investigations have served to strengthen this remarkable homology, which would have been impossible of demonstration but for the survival of a single class of vertebrates, the Cyclostomes, of which the Petromyzontidæ, or Lampreys, are the best known order. The lamprey embraces in its own life history both the fullest development of the endostyle mechanism[2] and the characteristic ductless

* Received for publication, December 30, 1912.

[1] I am indebted to Professor B. F. Kingsbury for the privileges of his laboratory and for helpful criticisms and suggestions. I wish also to thank Professor S. H. Gage for helpful suggestions, and Mr. J. F. Badertscher for aid in many technical problems.

[2] In addition to the ventral midline subpharyngeal glandular structure, or endostyle proper, there are accessory structures directly continuous with the endostyle epithelium developed in the pharyngeal mucosa that are believed to function as the path of conduction for the slime cord issuing from the orifice of the endostyle. This function has not, however, been observed in the Ammocoetes and is based on reasoning by analogy from Giard's (Giard, A., *Arch. de zoöl. expér. et gén.*, 1872, i, 525; 1873, ii, 481) and Fol's (Fol, H., *Morphol. Jahrb.*, 1874, i, 222) experiments with the endostyle structure of Tunicates, where they observed the cord of mucus issuing from the endostyle to pass forward, arch up around the sides of the pharynx to the dorsal midline, then pass back along this line to the esophagus. In the Ammocoetes the anterior part of this structure begins at the anterior margin of the large elliptical orifice of the

thyroid common to all Gnathostomes. The endostyle persists throughout the larval period of the animal's existence in full activity,[3] then at the metamorphosis, which was first made known to the zoölogical world by August Müller,[4, 5] the endostyle undergoes involution, all but a remnant disappearing, and other profound organic changes occur. This remnant assumes the form of ductless thyroid follicles and persists throughout the adult life of the lake lamprey (*Petromyzon dorsatus*) as a true ductless thyroid. It has been stated by some observers that the thyroid follicles may disappear entirely in old sea lampreys (*Petromyzon marinus*).

It is the purpose of this paper to record in some detail the series of changes that take place in the endostyle at metamorphosis and to

endostyle with which it is continuous and just posterior to the third gill arch pillar (figures 1a, 1b, and 1c). At the beginning it is a deep slit-like fold of the pharyngeal floor and roughly of the shape of an inverted T. Just anterior to the third pillar the groove widens and about the middle of the second gill pouch divides into right and left arms which continue forward with slight divergence to a point just anterior to the second gill arch pillar where the divergence becomes more marked. These grooves become shallower as they pass forward, outward, and upward around the branchial sac at its most anterior part to join the prominent dorsal midline fold where they fuse with the ciliated epithelium on the sides of this pendant-like fold and continue back along the midline to the esophagus. The ventral surface of this dorsal ridge or fold is covered with stratified squamous epithelium out of which the permanent esophagus will be formed. The posterior portion of the structure begins at the posterior lip of the endostyle orifice in the midline and extends backward along the midline of the pharyngeal floor as a shallow and narrow groove lined with ciliated epithelium continuous with that of the endostyle. This groove gradually gets smaller and narrower to end at the entrance of the larval esophagus.

The structure, then, that makes up this accessory endostylar mechanism is (1) the large, ventral midline duct-like ciliated groove issuing from the anterior lip of the gland orifice which divides into (2) two peripharyngeal ciliated grooves which terminate in the dorsal midline in the (3) ciliated dorsal ridge and (4) the shallow, narrow ventral midline groove from the posterior lip of the gland orifice to the esophagus.

[3] This persistence lasts at least for three, and possibly for four years, according to Gage (Gage, S. H., The Lake and Brook Lampreys of New York, Wilder Quarter-Century Book, Ithaca, 1893, 421).

[4] Müller, A., *Arch. f. Anat. u. Physiol.*, 1856, 323; *Ann. and Mag. Natural Hist.*, 1856, ser. ii, xviii, 298.

[5] According to von Siebold (von Siebold, K. T. E., Die Süsswasserfische von Mitteleuropa, Leipzig, 1863) this metamorphosis was known to Leonhard Baldner, a fisherman of Strassburg, in 1666.

ascertain what type or types of the endostylar epithelium persist as the lining epithelium of the ductless thyroid follicles, with the hope that it may serve as a step toward some clue to the thyroid function in this, which is seemingly the simplest form of the ductless thyroid.

THE NORMAL ANATOMY OF THE ENDOSTYLE.

The endostyle in the course of its development in the Ammocoetes acquires a complicated morphology which in section is not easily made out. On this account I have reconstructed the organ from a brook lamprey (*Lampetra wilderi*) in the collection of Professor S. H. Gage, of Cornell University[6] (Figures 1a, 1b, 1c, and 2).

This very prominent gland in the Ammocoetes lies just beneath the pharyngeal mucosa in the ventral midline and is contained within the cartilaginous framework of the branchial sac. It extends from the beginning of the first branchial pouch to the posterior border of the fifth branchial pouch and opens into the branchial sac by a large slit-like orifice just posterior to the third gill arch pillar. This orifice is situated in the ventral midline approximately at the middle of the long axis of the gland and is continuous with the posterior end of the deep pharyngeal groove. The gland is composed of right and left symmetrically opposed halves (figure 4). Each half consists of a single straight chamber anterior to the pharyngeal orifice (figure 3a). At the anterior border of the orifice each of the anterior chambers divides into an external and internal chamber (figure 3b). Each posterior arm contains approximately one half of the epithelial investment of the corresponding anterior chamber. The two external chambers extend directly posterior, and behind the posterior border of the coil of the internal chambers curve into the midline to extend a short way back side by side and end blindly just posterior to the bifurcation of the ventral aorta (figure 3c). The internal chamber of each half of the gland extends posteriorly side by side to a point approximately two thirds of the distance between the orifice and the posterior extremity of the two lateral chambers, then curves upward and forward, and reaches the vicinity of the gland orifice where it curves downward and backward and again upward

[6] The total length of specimen 15 T, fourteen months old, was 39 mm. The total length of the endostyle was 3.08 mm.

and slightly forward to end blindly (figures 1a, 1b, 1c, and 2). Thus it describes about one and one half elongated coils. These two internal or coiled chambers if extended would be longer than the two lateral chambers. I have spoken of these coiled internal chambers as if they were separate, but they are not strictly separate chambers since the septum is not complete. They are separate, however, in that they arise from opposite halves of the organ. The median septum is complete anterior to the orifice, while at the orifice it becomes partial and remains so throughout the extent of the internal or coiled chambers. The anterior chambers then communicate with each other only at the orifice. The two lateral chambers posterior to the orifice also communicate only at the orifice, and the two internal chambers communicate throughout their length on account of the incomplete median septum. All chambers communicate with each other at the orifice.

The gland is contained within a well formed fibrous capsule which anterior to the orifice is composed of an outer layer surrounding both halves and an inner layer which passes into the septum. Posterior to the orifice the outer fibrous investment of the whole organ is well developed, but the septa dividing the four chambers are very atrophic.

The ventral aorta is continued forward in the midline from the conus arteriosus as a single tube on the dorsal surface of the posterior extension of the lateral chambers until it reaches the posterior edge of the coiled inner chambers. Here it divides into two trunks which are continued forward on either side of the coiled chambers and on the dorsal surface of the lateral chambers to the anterior end of the endostyle where they terminate in the first pair of gill arteries.

The Lining Epithelia.—Anterior to the orifice the most striking feature of the interior of each chamber is the large visceral invagination, or infolding (flask-shaped in transverse section (figure 3a)), from the ventrolateral wall carrying blood vessels and stroma, but composed for the most part of four large bundles (fan-shaped in cross section) of large cuneiform cells, two on each side of the midline of the invagination. These fan-shaped columns extend the entire length of the organ and are composed of long wedge-like cells

with large nuclei lying at the base of the cells; *i. e.*, at the periphery of the columns. These long cells taper to a central core which opens on to the free surface by a small pyramidal projection. The finer cytology of these cells is not easily made out owing to the difficulty of obtaining good fixation. The nuclei are large, round, and vesicular. The cytoplasm also stains with basic dyes and one gets the impression that faintly staining granules and longitudinal striations are present, but, as Schneider remarks, this may be due to cytolysis. Each bundle is surrounded by a separate fibrous investment. These four bundles of gland cells make up the bulk of the interior of the invagination.

Covering the surface of the visceral invagination and thence reflected on to the septum and lateral wall of the chamber, one can distinguish four other types of epithelial cells. First on either side of the four small pyramidal openings of the large bundles there is a zone of narrow high columnar epithelium with long densely packed cilia. The nuclei of these cells stain densely, are much elongated, closely packed, and situated near the middle of the cell bodies. The cytoplasm stains with acid dyes. Extending up to the neck-like constriction of the visceral invagination is the second type of epithelium,—high columnar, with coarser and shorter cilia. These cells are of the same length as those just described, but are broader. The nuclei are round, pale, and vesicular, and lie in the basal third. The cytoplasm stains a pale red with eosin and in addition contains large quantities of a yellow flocculent and granular pigment. The knob-like apex of the fold anterior to the orifice is covered with a third type directly continuous with the second type and composed of low columnar cells with short coarse cilia. The cells have darkly staining elongated nuclei situated about the middle of the cell bodies, and the cytoplasm, while taking acid dyes, lacks the yellow pigment of the second type. This type is continuous at the orifice with that lining the pharyngeal grooves. A small zone of this same type of epithelium also occurs at the base of the visceral invagination on each side and merges into the fourth type of epithelium which covers the parietal walls, septum, and lateral wall. This area is covered with low cuboidal or flattened endothelial-like cells arranged in a single layer, as are also all the other epithelia.

At the orifice, as stated above, each anterior chamber divides so that half of the flask-shaped invagination of each anterior chamber continues in each of the four posterior chambers, the outer half of each invagination continuing as the lateral chambers, and the inner halves going into the internal, or coiled, chambers (figure 2). There is no change in the types of epithelia, each posterior chamber containing all the types found in the anterior chambers, but the number of cells is reduced approximately by half.

The glandular portion of the endostyle then contains five distinct types of epithelium: first, the four large bundles of cuneiform cells of each half of the gland; second, the narrow, columnar, densely nucleated and densely ciliated cells on each side of the pyramidal openings of the cuneiform bundles; and third, the broad columnar cells with round pale nuclei containing abundant yellow pigment granules in the cytoplasm. All these forms are confined wholly to the visceral invagination. The fourth form occurs on the knob-like apex of the visceral folds in the anterior chambers which at the orifice fuses with the parietal epithelium and at the angles of the base of the fold merges into the parietal epithelium. These cells are also columnar, coarsely ciliated, with dense elongated nuclei, and contain none of the pigment of the third type. The fifth type forms the parietal lining of the chambers, and is composed of a single layer of large endothelial-like cells.

These five types of epithelia are quite distinct and offer no difficulty of recognition by ordinary methods. I have given a detailed description of these five types because of their importance in the changes associated with the metamorphosis. In order to avoid repetition in the descriptions of the fate of each type of epithelium from the inception to the completion of metamorphosis, the five epithelia will be designated as types as follows (figure 5):

Type I.—The large fan-shaped bundles of cuneiform cells.

Type II.—The narrow densely nucleated and ciliated high columnar cells extending for a short distance on each side of the narrow openings of the cells of type I.

Type III.—The broad columnar ciliated epithelium with yellow granular pigment.

Type IV.—The low columnar ciliated cells occupying three sites: (1) the angle between the visceral fold and the parietal wall; (2*a*) the similar

angle at the apex of the visceral fold posterior to the orifice; and (*2b*) the knob-like free apex of the visceral fold anterior to the orifice.

Type V.—The flat epithelium lining the parietal walls and septum.

THE METAMORPHOSIS.

I have found no publications dealing specifically with the metamorphosis of the endostyle, although several observers have referred to it in a general way. Rathke[7] was the first to describe the endostyle of Ammocoetes as a gland, and in 1856 August Müller[8] observed that it disappeared at the metamorphosis. He thought that the structure became the muscular portion (tongue) of the sucking apparatus. Schneider[9] gives the first account of the metamorphosis of the endostyle as ascertained from two metamorphosing specimens of *Ammocoetes branchialis* given him by von Siebold. He noted that the large bundles of cuneiform cells disappeared during the change, while the connective tissue framework and lining ciliated epithelium remained. He stated that the fibrous tissue increases and forms a dense wall about the epithelium and gradually compresses it, although the epithelium holds to its orderly arrangement in the tube until at last the fibrous tissue growth cuts the tubules into segments and thereby obliterates all its original endostylar structure and creates the ductless thyroid follicles. W. Müller in his earlier paper,[10] while recognizing the thyroid gland in the adult *Myxine glutinosa,* seems to have mistaken the salivary gland for the thyroid in *Petromyzon fluvialis.* In a later paper[11] he states that only at metamorphosis the large glandular endostyle atrophies and only a small remnant of its epithelial lining remains as the ductless thyroid follicles of the sexually mature lamprey.

The extensive papers of Schneider's pupil, Kaensche,[12] and of Bujor,[13] give no account of the changes taking place in the endostyle at metamorphosis.

My observations are based on the study of twenty-four metamorphosing specimens taken from the mud and sand of Cayuga Lake Inlet at Ithaca, New York. Specimens for microscopic study were taken from the stream twice each week from June 17 to August 11, 1912.

Metamorphosing specimens in excess of those needed at the time of taking were stored in laboratory aquaria which were half filled with mud and through which running water was continually passing. After August 11 one of these laboratory specimens was removed

[7] Rathke, M. H., Bemerkungen über den inneren Bau des Bricke, Danzig, 1826.
[8] Müller, A., *loc. cit.*
[9] Schneider, A., *Ann. and Mag. Natural Hist.,* 1873, ser. iv, xi, 236.
[10] Müller, W., *Jenaische Ztschr.,* 1871, vi, 428.
[11] Müller, W., *Jenaische Ztschr.,* 1873, vii, 327.
[12] Kaensche, C. C., *Zoöl. Beitr. von A. Schneider,* 1890, ii, 219.
[13] Bujor, P., *Rev. biol. du nord de la France,* 1890–1, iii, 301; 1891–2, iv, 41.

every fifth day until September 20. I was able therefore to study an unbroken series from June 17 to September 20, under natural conditions until August 11, and modified by laboratory residence from August 11 to September 20.

The first specimen showing external signs of metamorphosis was taken on July 19. In a specimen taken July 16 and showing no external signs of metamorphosis, microscopic examination revealed in the endostyle changes that in the light of subsequent studies of the series as a whole must be considered as the earliest evidences of metamorphosis; namely, a thickening of the fibrous capsule and a slight reduction in the volume of the cells of type I. The specimen of July 19 shows, on careful examination, external changes. The dentaphores are visible as a stipple-like roughening on the inner surface of the upper leaflet of the hood and there is partial obliteration of the normal sharp angle at the juncture of the upper and lower leaflet of the buckle hood. The eyes could not be seen in the fresh specimen, but after a day's fixation in formalin, round whitish spots in the location of the eyes were visible through the skin. The prominent endostyle changes are the same as, but slightly more marked than those recorded for the specimen of July 16.

The specimen of July 23 shows obvious external changes. The body length is 14.7 cm., the eyes are clearly visible, the color is slate gray on the dorsal surface, and brownish yellow on the ventral surface. Dentaphores are visible, and there are slight elongation of the head and partial obliteration of the angles of the buccal hood.

The endostyle (figure 6) has undergone a striking reduction in size, measuring 1.006 mm. in diameter at the level of the orifice, as compared with 1.518 mm. for the normal endostyle of an ammocoetes of the same size. The fibrous capsule is sharply outlined as a thick band closely investing the gland and involving the septum as well. There is also a new formation of fibrous tissue in the periglandular zone. The interval between the gland and the pharyngeal mucosa is increased by this new tissue formation which is in the location of the future tongue. Of the epithelia, the cells of type I are reduced one third of their original volume, although they retain their wedge shape and arrangement. The nuclei of these cells have migrated from the base of the cells, *i. e.*, the periphery of the fan-shaped formation, and occupy the middle third of the cells. All four columns are affected to about the same extent. The cells of types II, III, and IV are somewhat compressed, but their general appearance is but little altered. The cells of type V have become more cuboidal owing to reduction in the size of the chambers.

The specimen of July 26 is 14.4 cm. in length. The eyes are distinct, the nasal pore large and patent, and the pineal eye white. The head and branchial region appear reddish and semitranslucent from the increased vascularity. The color on the dorsal surface is a dark slate gray and on the ventral surface it is tinged with yellow. The endostyle measures 0.627 mm. in diameter at the level of the orifice and exhibits reductions in the volume of all its component parts (figure 7). The sharp line of demarcation between the thickened capsule and the newly formed tissue external to it, which was a prominent feature in

the specimen of July 23, is absent and the two tissues merge. The whole area is much more vascular and contains large blood spaces. The tongue anlage is visible as a rounded bundle of large closely set elongated cells. The cells of type I are still detectable in the fan-shaped areas, although connective tissue has begun to invade them. The cell boundaries are for the most part lost. There is clumping of the cell masses. The nuclei are scattered irregularly through the area and present many stages of dissolution. The surface connection of these cell bundles is lost although the site of this connection is recognizable as slight notches in the cells of type II between which these bundles normally opened. The cells of type II are more compressed though still ciliated and high columnar. The nuclei of these cells while normally dense and closely set are through compression so closely packed that their limiting membranes are barely visible. The cells of type III have undergone a great reduction in size and probably also in number. The cilia are still present. The characteristic yellow pigment granules are more in evidence, probably as a result of a more rapid destruction of the cells than of the pigment. The cells of type IV are the least affected of all the types. There is possibly some slight reduction of their volume and a loss of cilia. The cells of type V, as a result of the further shrinkage of the capsule, are more cuboidal. Their positions are slightly altered and the first evidence of piling up and overlapping is seen.

The specimen of August 1 is 12.5 cm. in length. The eyes are large and clear and produce slight disc-like prominences on the sides of the head as in the adult. The color on the dorsal surface is a dark slate gray, and that on the ventral surface is tinged with yellow. The hood is much elongated. The sucking disc is now complete and all outlines of the larval hood are absent. The pharyngeal grooves can still be made out and the gland duct, much elongated, is still patent notwithstanding the extreme atrophic changes in the endostyle. The pericapsular tissues are vascular. The dense fibrous tissue of the thickened capsule, so evident in specimens of July 16, 19, and 23, has disappeared and the rather loose, palely staining fibrous tissue extends to the gland epithelium lining the chambers. The septum between the two halves of the endostyle is now a wide band of proliferating fibrous tissue separating the two halves as well as rotating their dorsal portions outward. At the level of the orifice the gland diameter is 0.462 mm. The individual chambers anterior to the orifice are about 0.2 mm. in diameter, or slightly smaller than the diameter of each ventral aorta at the same level.

The fan-shaped bundles of type I are no longer distinguishable as such. Connective tissue ingrowths from the base of the visceral fold and from the fibrous tissue within the fold itself have broken up these areas so that one sees only shrunken and distorted cell masses with or without visible nuclei (figure 8). These areas are, in general, lighter in color. The cells of type II are still ciliated, and are considerably reduced in length and extent. All evidence of the openings of the cells of type I is gone. The nuclei are basal, very dark, and closely packed. The cells of type III are much reduced in size and number. The yellow pigment granules are relatively more abundant and are contained for the most part in the basal portion of the cell bodies, but are also present above the nucleus. The scattered cilia are still to be made out on these cells. The cells of type IV are well preserved although the cilia are absent. Their

extent is slightly reduced by compression. Their nuclei are large, darkly staining, and closely packed. The cells of type V, owing to the great contraction of the chambers, are irregularly distributed, piled up, in places two or more in thickness, and their nuclei show many stages of degeneration.

The specimen of August 5 is 14.9 cm. in length. The anterior portion of the endostyle is separated from the pharynx by the now large muscular anlage of the tongue which lies between the aortæ and the endostyle. The duct is elongated, and while its outline is visible, its pharyngeal connection has been closed at the level of the tongue anlage. All the pharyngeal grooves are gone and, in cross section, the branchial sac is a smooth walled oval chamber with the permanent esophagus anlage embedded above it in the dorsal midline. The endostyle chambers are still discernible in both the anterior portions as clefts, about 20 μ in width. A capsule as such is no longer visible. The fibrous tissue has become uniform so that no line of demarcation exists between the visceral fold and the parietal wall. The diameter of the endostyle at the level of the duct is 0.146 mm. All the epithelium of type I has been absorbed. The cells of type II have either atrophied beyond recognition or fused with the lower area of type IV. It appears as if this epithelium had gone, but I lack sufficient gradations to exclude a fusion. The cells of type III are now represented by a few atrophic cells containing a great abundance of yellow pigment granules (figures 9 and 10). The cells of type IV remain prominent. They are more cuboidal and their nuclei are probably paler than in previous stages. The cells of type V appear as an extruded mass of large, distorted, and disintegrating cell masses lying to the outer side of the cleft-like remnant of the chambers.

The specimen of August 9 is 12.5 cm. in length. All evidences of the true endostyle are absent in this specimen. The duct is obliterated. The small follicles and nests of epithelial cells lie in a rather uniform area of connective tissue. These follicles and cell nests are grouped into right and left bundles corresponding to the right and left halves of the old endostyle, and by the obliteration of the septum and ingrowth of fibrous tissue they are relatively widely separated. Anterior to the old orifice there are three to four follicles or nests of cells in each group corresponding to each half of the endostyle; while posterior to the old orifice and corresponding to the coiled portion of the inner chambers the follicular and cell nest remnants of the two halves of the old endostyle are closely approximated, and one can make out as many as twelve to fifteen follicles or cell nest areas. Of the epithelia, type I is completely absent, and type II is either completely absorbed or, becoming atrophic, has fused with the lower area of cells of type IV. Type III is still recognizable as a few nuclei surrounded by clumps of yellow pigment, in some levels extruded toward the lumina of the future follicles and in others pushed backward in the connective tissue stroma. Type IV is now the only prominent epithelium. The upper or dorsal area has in places assumed definite follicular shapes, utilizing the remnant of the original chamber as their lumina. In places the epithelial layer is slightly curved while in others it has completed the formation of the follicle. The lower mass of epithelium of type IV and possibly the fused cells from type II appear as a more or less curved layer of well preserved cuboidal cells surrounding the remnant of the original chambers. The cells of type V may still

be made out as a small mass of more brightly staining cell remnants in the connective tissue opposite the concavity of the cell layers of type IV (figures 11, 12, and 13).

From this stage on, the further involution is largely a matter of the absorption of the remnants of the disappearing types of epithelia, the completion of the follicle formations out of the persisting type of epithelium (type IV), the development of the tongue and of the branchial sac or bronchus, the formation of the great blood spaces about the tongue through the absorption of the connective tissue, and the rotations and possible migrations, distortions, and secondary constrictions of the original follicular tubes by changes in the interfollicular stroma.

EXPERIMENTAL OBSERVATIONS.

Experiment 1.—September 4, 1911. Sixty-five specimens were collected and provisionally classified as follows:

9 of the hatch of 1911, measuring 1.9 to 2 cm. in length.
34 of the hatch of 1910, measuring 6 to 6.5 cm. in length.
20 of the hatch of 1909, measuring 11 to 12 cm. in length.
2 of the hatch of 1908, measuring 14 to 15 cm. in length (metamorphosis complete).

The specimens were taken to Cleveland and placed in jars half filled with mud and sand in which some hay was placed, and running lake water was supplied continuously to each jar. On September 10 the twenty specimens of the hatch of 1909 were divided into four lots of five each and placed in separate jars. To two of the lots one drop of Lugol's solution was added daily, while the remaining two lots were kept as controls. On the 14th of each month, beginning September 14, 1911, and ending June 14, 1912, a specimen from the lots exposed to iodin and one of the controls were taken for histological examination. The object of this experiment was to see whether iodin might influence the endostyle structure and possibly afford a chemical connecting link with the ductless thyroid gland which is so markedly influenced by iodin. The results were entirely negative as regards any morphological change. This negative evidence, however, in so far as it is of value, supports the hypothesis of Müller, Dohrn, and others, based on morphological changes, that the physiology of the endostyle is quite different from that of the ductless thyroid.

Experiment 2.—Attempts were made to destroy the endostyle organ in ammocoetes varying in length from 10 to 16 cm., (1) by means of injecting paraffin into the gland lumen, and (2) by cauterizing with a fine electric needle. Ether vapor was found to produce a safe and quick anesthesia from which the animals rallied in a few minutes. Chloroform vapor and several solutions of chloretone and magnesium sulphate were tried, but proved to be too toxic.

The operation was performed by placing the anesthetized ammocoetes with its ventral surface up in a grooved pad of water-soaked absorbent cotton. For the paraffin injections, a fine hypodermic needle with screw adjustment for graduating the pressure was used. A mixture of paraffin oil and 52° paraffin was tried. The method was abandoned on account of the hardening of the paraffin in the needle as soon as it came in contact with the cold surface of the ammocoetes.

With the fine electric needle and the animal in the dorsal position the following procedure was carried out. The skin and musculature were burned through in the ventral midline between the second and fourth gill openings and the tip of the cautery was allowed to extend into the branchial sac. The entrance of the cautery into the sac is easily detected. The cautery was then extended slightly forward and backward from this point. This produced some destruction of the endostyle between the right and left branches of the ventral aorta in the region of the gland orifice. Ten ammocoetes were thus operated upon on July 13. All recovered and were kept until August 11, when the experiment was interrupted. The ammocoetes were all vigorous and active at the time they were killed. The cautery wounds had healed in all and the sites were marked by white, non-pigmented scars.

Histological examination of these ten specimens shows that the endostyle was only partially destroyed in each. The large bundles of cuneiform cells (type I) were destroyed at the cauterized site and exhibited no evidence of regeneration. There was an inflammatory reaction in the visceral invagination extending for a considerable distance beyond the cauterized area and containing eosinophiles. In none of the specimens were the chambers destroyed, as I hoped they might be, and it appears that an actual regeneration of at least two types of epithelium takes place: type IV, or that lining the duct and continuous with the pharyngeal grooves, and also type V, or the endothelial-like lining of the parietal walls. There is no evidence that the yellow pigment-bearing cells of type III regenerate. The duct was not destroyed in any of the specimens, although in two specimens evidence of severe injury was present, yet the epithelial lining had regenerated.

These observations, although meager, show that gross injury may be induced experimentally without death of the animal, and that some of the endostylar epithelia have little or no regenerative capacity, while others exhibit considerable regeneration within the period of a month. Further experiments extending over a longer time so as to include the effects of metamorphosis on animals which have been operated on in this way seem highly desirable.

SUMMARY.

The first specimen of *Ammocoetes branchialis* that showed histologically any atrophic changes in the endostyle was taken on July 16. These changes proceeded relatively rapidly for about a month, after which the endostyle as such was no longer recognized. All specimens examined after August 15 showed in cross section the characteristic ductless follicles more or less completely formed. More gradual and minor changes in the way of further absorption of cell remnants and completion of the follicles continued at least until September 1. Two specimens taken from the creek on September 4, 1911, showed complete follicle formation with some stainable colloid (figures 14 and 15). There was still yellow granular pig-

ment in the fibrous tissue between the follicles. In two specimens[14] taken on October 14, 1909, the pigment was absent and the follicles were more closely set, larger, and contained homogenous colloid. In the twenty-four specimens of ammocoetes studied, there were variations in the time of the onset of metamorphosis. There may also be variations in the rate of progress of the changes in different specimens. There is no evidence that removal of the animals from their native environment to the laboratory either increases or decreases the rate of metamorphosis. Schneider states that he was unable to get specimens kept in the laboratory to undergo metamorphosis. Gage, however, has repeatedly observed the metamorphosis under laboratory conditions, and the six of our specimens kept in the laboratory—some for forty days—remained in excellent condition and the metamorphosis proceeded as well as in those living in the creek. I know of no observations bearing on the question as to whether the metamorphosis may be hastened or delayed as it can be in tadpoles and other amphibia. It is probable, however, that physical conditions greatly influence the transformation.

These observations as to the length of time from the inception to the completion of metamorphosis indicate that a month and probably longer is necessary for the lake and brook lampreys of Central New York. This is in agreement with the observations of Gage[15] and of Müller[16] on metamorphosis in general, but is at variance with the views of Bujor,[17] who states that the process takes place within three to four days.

The first endostylar changes are a gradual shrinkage in the whole organ with thickening of the capsule and septum and proliferation of the connective tissue in the periendostylar zone. The tongue anlage is developed in this thickening just dorsal to the endostyle and anterior to the gland orifice. The size of the chambers progressively decreases and with the thickening of the septum the halves of the endostyle are both absolutely and relatively more separated.

[14] These two specimens were presented to me by Professor Kingsbury for serial sections.

[15] Gage, S. H., *loc. cit.*

[16] Müller, A., *loc. cit.*

[17] Bujor, P., *loc. cit.*

All the five types of epithelia are affected, the first to show the change being type I, the four fan-shaped bundles of cuneiform cells of each half of the endostyle. These disappear totally quite early. The next type to show marked changes is type III, or the cells with yellow pigment granules. Here the change is progressive and these cell groups in different stages of atrophy may be traced through to the fully developed follicles. The epithelium of type V, or the endothelial-like lining of the parietal walls of the chambers, is piled up and extruded laterally as the chambers contract or shrink. These cells in different stages of atrophy may be followed until the metamorphosis is nearing completion. It is certain that the cells of types I, III, and V play no part in the formation of the ductless follicles. With types II and IV the question is not so easily settled as it is from one or the other or from both of these types that the permanent follicles arise. One can say definitely that type IV plays the major rôle, but whether the cells of type II after fusion with the basal group of type IV do not also share in the formation of the ventral follicle of the given chamber, I cannot decide, but from the evidence obtainable this seems probable. It is significant that the cells of type IV are continuous with, and indistinguishable from, the cells lining the orifice and are continued anteriorly in the deep pharyngeal groove and peripharyngeal grooves as well as posteriorally from the orifice in the small pharyngeal groove. As to the fate of this extraglandular epithelium of type IV I have no data save that with the closing of the orifice and the formation of the permanent branchial sac these grooves with their ciliated epithelium disappear and the whole sac comes to be lined with plain stratified epithelium. The fact that the cells of the pharyngeal grooves and the lining cells of the gland orifice are continuous with the cells of the endostyle from which the permanent thyroid follicles are formed is not without significance in relation to the development of the thyroid of the higher chordates. One or more very large follicles are formed from the lower portion of this orificial epithelium of type IV.

Four ductless follicles are the maximum number that may be formed primarily in each half of the endostyle from the four areas of epithelium of type IV. From the specimens studied this maxi-

mum is frequently not obtained. Posterior to the orifice where four chambers exist, each corresponding to one half of an anterior chamber, but two follicles may be formed from each chamber, but in the coil these are proportionately increased, in cross section. Most of the detailed studies here recorded have been made on the part of the endostyle posterior to the coil where the simplest conditions exist. Here two follicles are ordinarily formed from each chamber.

In cross sections the follicles are at first only long tubules whose cavities are the remnants of the original endostyle chambers, but when the metamorphosis is completed each of these primary tubules is cut up into several elongated closed sacs corresponding to the true ductless follicles of all higher chordates.[18] New follicles also arise by budding from these primary ones, and this process is probably of normal occurrence at the metamorphosis.

[18] Dr. H. D. Reed, of the Department of Zoölogy of Cornell University, gave me twelve specimens of adult lake lampreys taken at spawning time during the spring of 1912 from the Cayuga Lake Inlet. In examining histologically the thyroid areas of these sexually mature lampreys certain features worthy of mention were noted. (1) The thyroid area still shows the division into the right and left halves corresponding to the endostyle halves. This separation is most marked in the anterior half of the thyroid area. (2) The increase in number of follicles due to the coil of the inner chambers of each half of the endostyle can be made out. (3) In two specimens the follicles were much larger and the number of follicles was increased above that which obtains just after the metamorphosis, suggesting that both hypertrophy and hyperplasia of the epithelium lining the ductless follicles have occurred. This epithelium was originally that of type IV of the endostyle. This is of interest in suggesting that these cells, after involuting to a given level as part of the destruction of the endostyle, assume a resting stage from which they may again undergo hypertrophy. (4) In one of the twelve specimens (a large adult female) the thyroid follicles were very large, some measuring 0.214 mm. in diameter, although there was no increase in the number over that obtaining just after the metamorphosis. The lining epithelium was very high columnar and for the most part, if not completely, ciliated. The nuclei of these very large high columnar cells were large, rounded, and palely stained. In each follicle there was a homogeneous red staining colloid-like mass. The significance of this observation is probably greater than one can estimate from the few facts obtainable from this accidental specimen. The fact that the endostylar epithelium from which these follicles arise is ciliated, and that these cilia usually disappear rather early in the metamorphosis, suggests that some physiological factor is involved either by way of preventing normal involution through sustained physiological activity of the larval cells, or by inducing a regeneration of the involuted cells to the larval type.

EXPLANATION OF PLATES.

PLATE 66.

FIG. 1a. Photograph of a wax model with the pharyngeal mucosa attached, showing (*a*) the pharyngeal opening of the endostyle, (*b*) deep anterior pharyngeal groove, (*c*) branching of the anterior groove into the two peri-pharyngeal groves, (*d*) shallow posterior pharyngeal grooves, (*e*) left terminal branch of the aorta and (*f*) opening into branchial pouch.

FIG. 1b. Photograph of a wax model with the pharyngeal mucosa removed (dorsal view), showing (*a*) endostyle orifice, (*b*) coiled internal chambers, (*c*) left external chamber, (*d*) median raphe and septum, (*e*) conus arteriosus, (*f*) bifurcation of the aorta, and (*g*) gill arch artery.

FIG. 1c. Photograph of a wax model with the pharyngeal mucosa removed (lateral view).

FIG. 2. Photograph of transverse sections of the model, (*a*) anterior to the orifice, showing visceral invagination and the arrangement of the large bundles of cuneiform cells, (*b*) just posterior to the orifice, (*c*) through the coiled portion, and (*d*) through the posterior edge of the coil.

PLATE 67.

FIG. 3a. Photomicrograph of a transverse section of the endostyle anterior to the orifice.

FIG. 3b. Photomicrograph of a transverse section of the endostyle through the orifice.

FIG. 3c. Photomicrograph of a transverse section of the endostyle posterior to the coiled chambers.

PLATE 68.

FIG. 4a. Schematic diagram of the left half of the endostyle. The single anterior chamber branches at the anterior edge of the orifice into an inner coiled chamber and an outer straight chamber. In the diagram the inner chamber is distorted inward and rotated to the left from its normal upright position.

FIG. 4b. Composite diagram of the right and left halves with the two coiled chambers rotated outward from their normal upright positions.

FIG. 4c. Schematic diagram of the right half of the endostyle.

FIG. 5. Camera lucida outline of a transverse section of the left external chamber posterior to the coil, showing the arrangement and distribution of the five types of epithelium in the normal fully developed endostyle. × 107½.

FIG. 6. Camera lucida outline of a transverse section of the left external chamber posterior to the coil, showing the earliest changes in the epithelia at metamorphosis. × 367½.

PLATE 69.

FIG. 7. Camera lucida outline of a transverse section of the left external chamber posterior to the coil, showing a slightly later stage of metamorphosis. × 367½.

FIG. 8. Camera lucida outline of a transverse section of the left external chamber posterior to the coil, showing a slightly later stage of metamorphosis. × 367½.

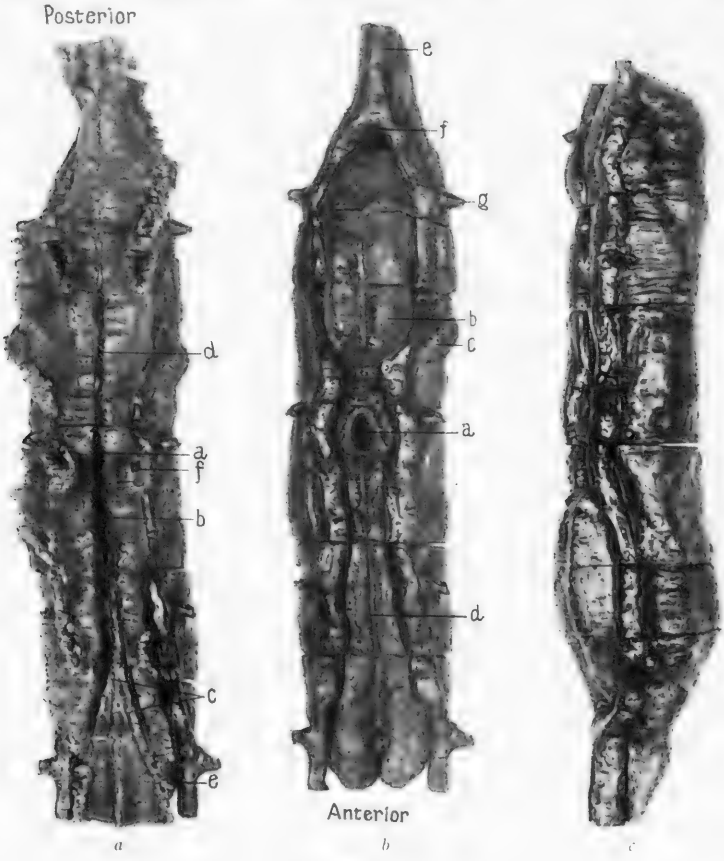

FIG. 1.

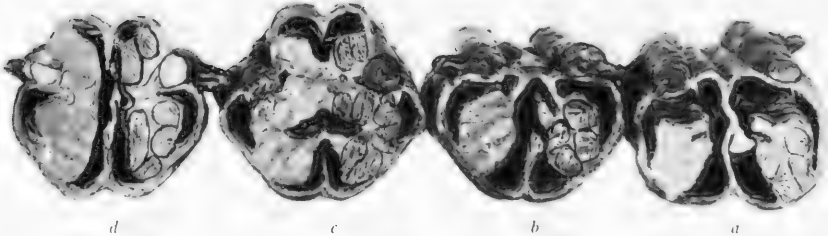

FIG. 2.

(Marine: Metamorphosis of Endostyle of *Ammocœtes branchialis*.)

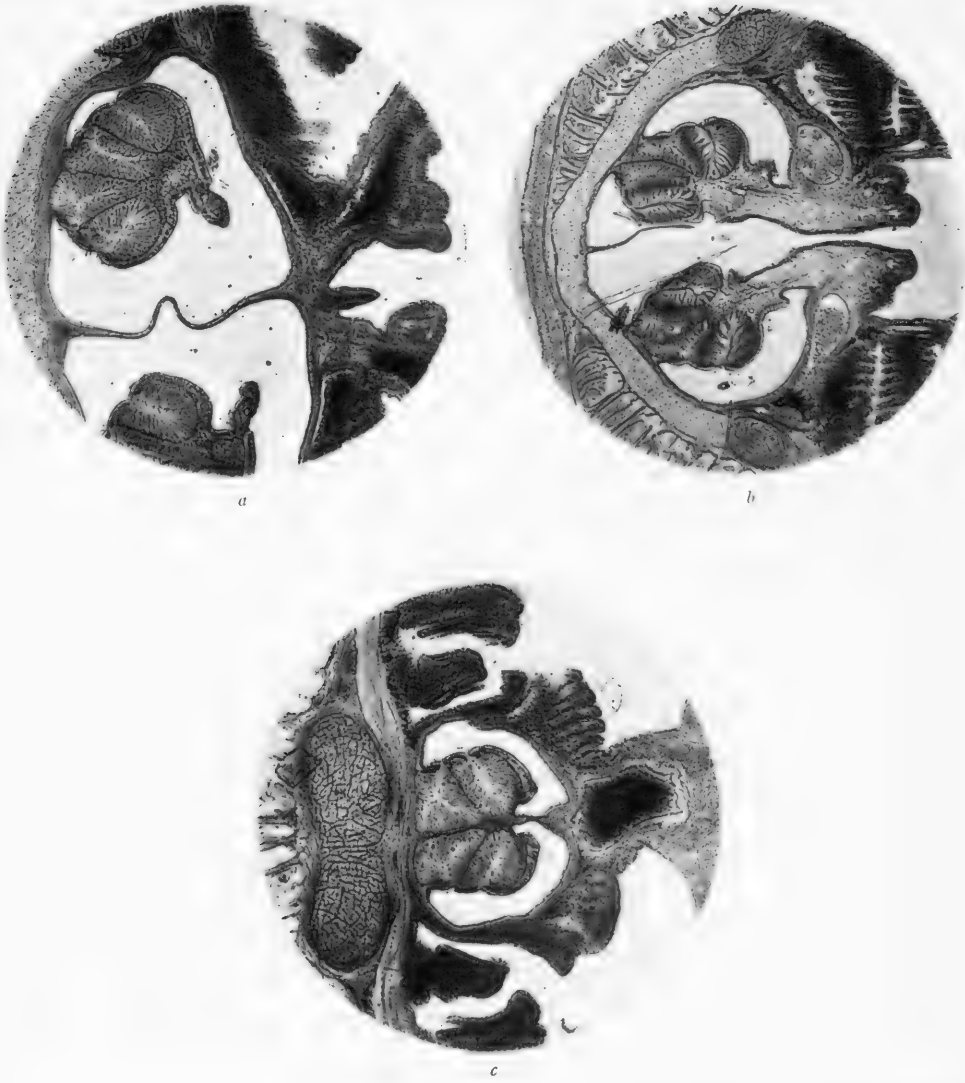

FIG. 3.

(Marine: Metamorphosis of Endostyle of *Ammocœtes branchialis*.)

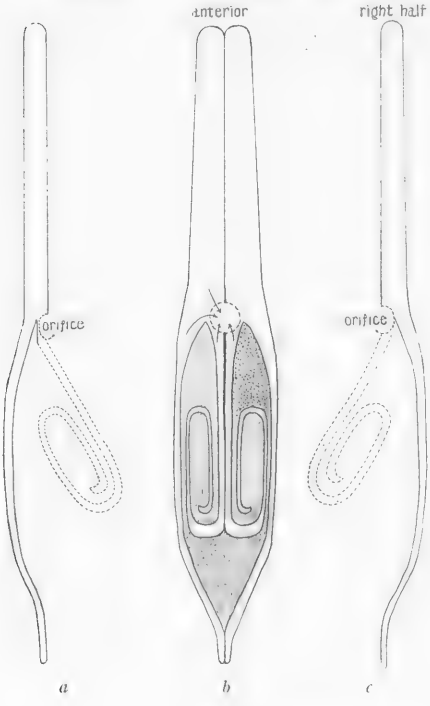

FIG. 4.

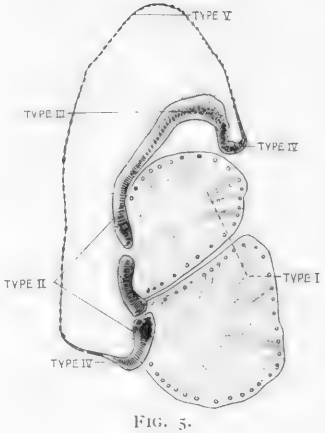

FIG. 5.

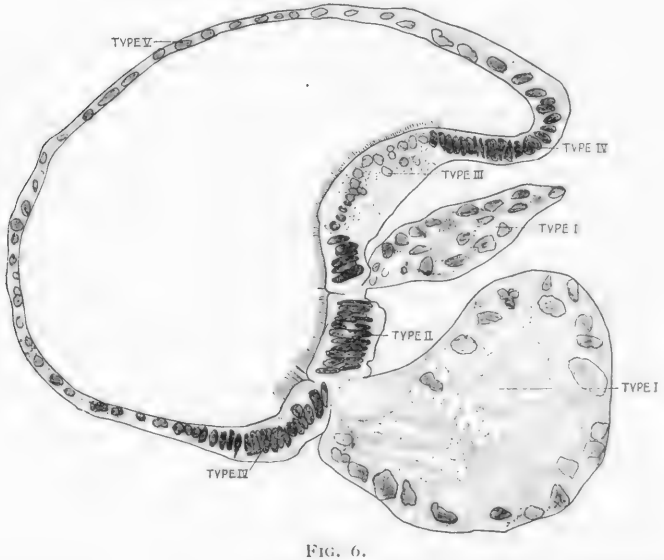

FIG. 6.

(Marine; Metamorphosis of Endostyle of *Ammocœtes branchialis*.)

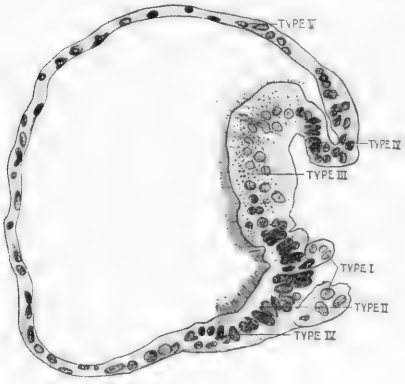

FIG. 7.

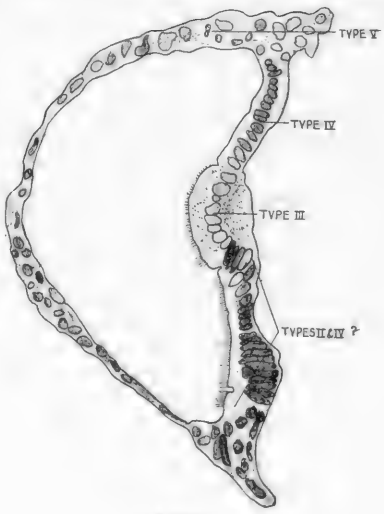

FIG. 8.

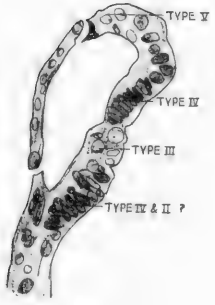

FIG. 9.

FIG. 10.

FIG. 11.

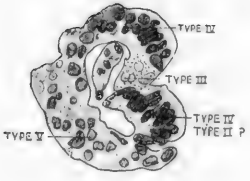

FIG. 12.

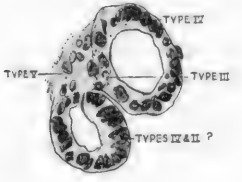

FIG. 13.

Marine: Metamorphosis of Endostyle of *Ammocœtes branchialis*.

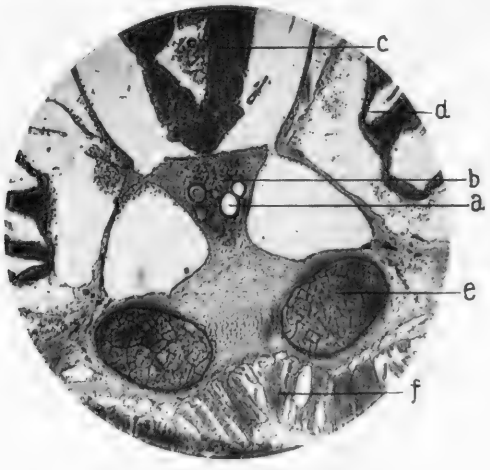

FIG. 14.

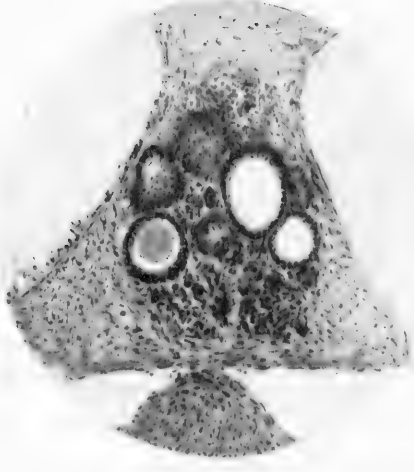

FIG. 15.

(Marine: Metamorphosis of Endostyle of *Ammocœtes branchialis*.)

FIG. 9. Camera lucida outline of a transverse section of the left external chamber posterior to the coil, showing a still later stage of metamorphosis. × 367½.

FIG. 10. Camera lucida outline of a transverse section of the left external chamber posterior to the coil, illustrating the last stages of the metamorphosis. × 367½.

FIG. 11. Camera lucida outline of a transverse section of the left external chamber posterior to the coil, illustrating a slightly later stage of the metamorphosis than is shown in figure 10. × 367½.

FIG. 12. Camera lucida outline of a transverse section of the left external chamber posterior to the coil, illustrating the earliest ductless thyroid formation. × 367½.

FIG. 13. Camera lucida outline of a transverse section of the left external chamber posterior to the coil, illustrating two distinct follicle formations from the single chamber. × 367½.

PLATE 70.

FIG. 14. Photomicrograph of a transverse section of the thyroid area from a specimen removed from the creek on September 4, 1911, in which the metamorphosis was complete, showing (*a*) the thyroid area with complete follicle formations, (*b*) pigment and cellular debris, (*c*) tongue, (*d*) gills, (*e*) cartilaginous framework of the branchial sac, and (*f*) somatic musculature.

FIG. 15. Photomicrograph of the thyroid area shown in figure 14. Higher magnification.

ON THE POSSIBLE IMPORTANCE OF COLLOIDAL PROTECTION IN CERTAIN PHASES OF THE PRECIPITIN REACTION.*

By HANS ZINSSER, M.D., and STEWART W. YOUNG.

(*From the Laboratories of Stanford University, California.*)

It has been frequently observed by those who have worked with the precipitin reaction that the sera of protein-immunized animals may contain, at certain times, both precipitin, and, side by side with it, unassimilated remnants of the antigen. Such sera, when taken, are clear, but will show precipitation not only when mixed with dilutions of the antigen, but also when added to other homologous precipitating sera. This phenomenon has been noticed by Linossier and Lemoine (1), Eisenberg (2), Ascoli (3), and others, and has been extensively studied by von Dungern (4). Gay and Rusk (5) have recently observed it in connection with the rapid method of precipitin production of Fornet and Müller (6), and have noted that such sera, although containing both antigen and precipitin, do not possess complement-fixing properties. According to Uhlenhuth and Weidanz (7), the antigen may persist in the sera of protein-immunized animals, in demonstrable amounts, as long as fifteen days after the last injection, and it is constantly present during this period, but in progressively diminishing amounts.

We are thus confronted by the apparently paradoxical phenomenon of the presence in these sera, side by side, of an antigen and its homologous precipitin, incapable of reacting with each other, although each of them readily reacts with precipitin or antigen respectively, when these are added from another source.

Many attempts have been made to account for this. A number of observers, notably Eisenberg, have concluded, from extensive analyses of quantitative relationships, both of agglutinin and precipitin reactions, that these take place according to the laws of mass action. In consequence, in addition to the combined precipitin-anti-

* Received for publication, December 30, 1912.

gen complex present in all mixtures of the two, there should also be present free dissociated fractions of each, in amounts dependent upon relative concentrations. This might explain conditions such as those described above.

von Dungern, whose paper forms one of the most extensive studies of the phenomenon with which we are concerned, does not believe that precipitin reactions can follow the laws of mass action, and explains the simultaneous presence of precipitin and antigen in the same serum by assuming a multiplicity of precipitins.[1] He believes that every proteid antigen contains a number of related partial antigens which give rise in the immunized animal each to a partial precipitin. In sera in which both antigen and precipitin are found side by side and free, he believes that the antigen is of a nature that has no affinity for the particular partial precipitin present with it. He says:

"Auch hier handelt es sich nicht um zwei reaktionsfähige Körper, deren Verbindung aus irgend welchen Gründen unterbleibt, sondern um Substanzen, welche keine Affinität zueinander besitzen. Die betreffenden Kaninchen haben zu dieser Zeit noch nicht alle möglichen Teilpräzipitine gebildet, sondern nur einzelne derselben. Diese zunächst produzierten, nur auf bestimmte Gruppen der präzipitablen Eiweisskörper passenden Partialpräzipitine sind es, welche nach der Absättigung aller zur Verfügung stehenden zugehörigen Gruppen der präzipitablen Substanz im Serum nachweisbar werden. Daneben bleibt aber ein anderer Teil der präzipitablen Substanz, der keine Affinität zu dem gebildeten Präzipitin besitzt, bestehen, solange bis ein anderes Partialpräzipitin von den Kaninchenzellen geliefert wird, welches sich mit Gruppen der in Lösung gebliebenen Eiweisskörper vereinigen kann."

The phenomena described above have been observed by the writers in a number of sera, and the facts recorded by others have been confirmed. It does not seem to them, however, that the explanations so far advanced are compatible with all the experimental data, and it is the main purpose of this paper to offer an explanation which, they believe, meets all the observed facts. Since the serum experiments on which this opinion is based are largely in accord with the published experience of other workers, these experiments will be detailed in the case of a few sera only, in which a systematic series of observations were made.

[1] A number of sera in which both antigen and precipitin were simultaneously present were noticed, incidental to other work, by one of the writers in the laboratory of Professor Ulrich Friedmann in Berlin. A detailed study of the conditions was taken up at Stanford University during the fall of 1912.

SERUM EXPERIMENTS.

Sera A and B were obtained from two rabbits injected intravenously with horse serum on three consecutive days and bled on the seventh day after the last injection.

Titrated for their precipitin contents against horse serum, they gave the following results:

Horse serum, 0.5 c.c.	Serum A, 0.5 c.c.	Serum B, 0.5 c.c.
1 : 50	+ + +	+ + +
1 : 100	+ + +	+ + +
1 : 200	+ + +	+ + +
1 : 500	+ +	+ +
1 : 1,000	±	+ +
1 : 2,000	—	—
1 : 5,000	—	—

They were then tested for the presence of antigen remnants by means of serum 1, obtained from a rabbit immunized with horse serum by a less rapid method, in which no antigen could be demonstrated, by means of a fourth precipitating serum, and which precipitated horse serum in dilutions of 1 : 4,000.

The results of this were as follows:

Serum A.		Serum 1.	Precipitate.
0.3 c.c.	+	0.3 c.c.	+
0.3 (1 : 2)	+	0.3 c.c.	+ +
0.3 (1 : 5)	+	0.3 c.c.	+ + +
0.3 (1 : 10)	+	0.3 c.c.	+ +
Serum B.		**Serum 1.**	**Precipitate.**
0.3 c.c.	+	0.3 c.c.	+ + +
0.3 (1 : 2)	+	0.3 c.c.	+ +
0.3 (1 : 5)	+	0.3 c.c.	+
0.3 (1 : 10)	+	0.3 c.c.	±

On mixing sera A and B a slowly forming but distinct precipitate was noticed. This precipitate was noticeable as turbidity within twenty minutes and, after twelve hours in the refrigerator, settled out in flakes. The same observation has been recorded by Schmitt and is cited by Uhlenhuth as a warning against the use of mixtures of precipitating sera in making forensic tests. No similar precipitation was observed when other precipitating sera not containing antigen were mixed. Thus:

Serum A.		Serum B.	Precipitate.
0.3 c.c.	+	0.3 c.c.	+ + slow in forming.

Serum 1.		Serum 2.	Precipitate.
0.3 c.c.	+	0.3 c.c.	None.

Sera A and B were then examined for possible complement-fixing properties. The complement used in this test was titrated just before use with the same sensitized sheep corpuscles used in the experiment, and 0.05 of a cubic centimeter was determined as the smallest amount which would produce complete hemolysis within one hour. The experiment was as follows:

1. Serum 1 (control)	0.4 c.c. + 0.05 complement + 3 c.c. salt solution	1 hr. in incubator.
2. Serum 2 (control)	0.4 c.c. + 0.05 complement + 3 c.c. salt solution	
3. Serum A	0.4 c.c. + 0.05 complement + 3 c.c. salt solution	
4. Serum B	0.4 c.c. + 0.05 complement + 3 c.c. salt solution	

After one hour in the incubator sensitized cells were added with the following results:

1. + sensitized cells = complete hemolysis in 1 hr.
2. + sensitized cells = complete hemolysis in 45 min.
3. + sensitized cells = complete hemolysis in 45 min.
4. + sensitized cells = complete hemolysis in 40 min.

It is seen that none of these sera fixed complement, although they were not heated to destroy possible anticomplementary substances which are known to form in many normal sera on standing. Had this occurred we had planned to repeat the test with the heated sera but the fact that the complement fixation tests were carried out within a few days after the blood was taken from the rabbits probably was the cause of our avoiding this possible source of confusion.

The observations shown in the above protocols were made incidently to other work, and the sera A and B were set away in the refrigerator for a little less than four weeks without being examined. At the end of this time it was noticed that moderate precipitates had settled out in both tubes. Titration at this time showed that both antigen and precipitin were still present in the sera, but that the precipitin contents had diminished from those stated above, in the previous titration, to a limit of 1:100 in the case of A and of 1:200 in the case of B. Even in the higher concentrations of horse serum,

moreover, the precipitates which appeared were in no case heavy or flocculent. Since our previous titration of the antigen contained in these sera was very incomplete, it was unfortunately not possible to make a reliable estimate of the relative diminution of antigen in the sera.

Complement fixation tests done with the precipitate found in tube A showed slight but distinct fixation of quantities of complement less than 0.05 of a cubic centimeter. Thus:

1. Precipitate (tube A) + 0.05 c.c. complement (in 1 c.c. salt solution).
2. 0.05 c.c. complement (in 1 c.c. salt solution).
3. 0.025 c.c. complement (in 1 c.c. salt solution).

These tubes were placed for one hour at 37° C. and sensitized sheep cells were then added.

The results were as follows:

1. Slight hemolysis in 1 hr.; incomplete over night (about half).
2. Almost complete in 1 hr.; complete over night.
3. About like tube 1.

To sum up, then, the experiments show again that antigen and precipitin may be present side by side in the same serum; that such sera do not by themselves fix complement; that such sera may be precipitated both by the addition of specific precipitin and by mixture with the homologous antigens; that on being added, one to the other, such sera, if homologous, may precipitate each other; and, finally, that, on standing, spontaneous precipitation with a loss of precipitin may take place. Furthermore, the precipitate thus formed has slight but distinct complement-fixing properties.

The spontaneous precipitation of sera in which both antigen and antibody are present has been noticed not only by us, but is mentioned as a frequent occurrence by Uhlenhuth and Weidanz, and others. These writers consider the possibility of slow autoprecipitation which, for some reason, has been delayed. Our observation that within a month at refrigerator temperature a considerable loss of precipitin accompanied the formation of the precipitate, makes this seem likely, as does the fact that the precipitate thus formed possessed slight complement-fixing properties. On the other hand,

it must be admitted that normal sera may occasionally precipitate spontaneously. Uhlenhuth mentions this as possibly invalidating the assumption that such autoprecipitation represents a late specific union of antigen and precipitin. However, the spontaneous precipitation of normal sera is very irregular, is not clearly understood, and may, as Uhlenhuth suggests, depend on various fortuitous circumstances. And it seems clear from the investigations of Merkel (8), as well as from our own experience, that late spontaneous precipitation occurs with great frequency in protein-precipitating sera, and more especially in those which have been taken from the animals too soon after the last injection, when free antigen in considerable amounts has been shown to be present. It seems to us, moreover, that the irregularity of this occurrence lends a support to the explanation we have to offer, since, as we shall see, this depends upon a very delicate quantitative adjustment of the reagents taking part in the precipitation.

Bearing upon this point, and corroborating our previous experiments, are observations which we have recently made with the sera of three rabbits immunized with human ascitic fluid. One of these sera showed slight but distinct turbidity within forty-eight hours after being taken from the rabbit, and very slight precipitation, not due to bacterial growth, after seventy-two hours.

The three rabbits of this series were treated as follows:

1. A received three injections of human ascitic fluid, 2 c.c. each, at three day intervals, and was bled on the sixth day after the last injection.

2. B received three injections of the ascitic fluid, 3 c.c., 3 c.c., and 4 c.c., at three day intervals, and was bled eight days after the last injection.

3. C received three similar injections of 5 c.c. each on three successive days and was bled on the fourth day after the last injection.

Titrated for their contents of precipitin, A precipitated the ascitic fluid in dilutions of 1 to 500, B in dilutions of 1 to 2,000, and C in dilutions of 1 to 100, giving very slight turbidity in dilutions of 1 to 200.

Added one to another, these sera gave mutual precipitation, which was slow, not appearing within one hour in the incubator, but showing flocculent precipitation after twelve hours in the ice chest. The relative amounts of the precipitates formed were as follows:

1. 0.5 c.c. A + 0.5 c.c. B = + + moderate precipitate.
2. 0.5 c.c. B + 0.5 c.c. C = + + + + heavy.
3. 0.5 c.c. A + 0.5 c.c. C = + a little less than tube 1.

The precipitates formed in these tubes were once washed in salt solution and found to fix completely 0.025 of a cubic centimeter of complement, an amount sufficient to hemolyze the control tubes entirely within seventeen minutes at 37° C. The red cells in these fixation tests were saturated with sensitizer by the addition of an excess afterwards removed by washing. A and C were titrated for their antigen contents by means of B, with the following results:

B + A	Precipitate.	B + C	Precipitate.
0.3 c.c. + 0.3 c.c. (1: 2)	= +	0.3 c.c. + 0.3 c.c. (1: 2)	= + +
0.3 c.c. + 0.3 c.c. (1: 5)	= +	0.3 c.c. + 0.3 c.c. (1: 5)	= +
0.3 c.c. + 0.3 c.c. (1:10)	= ±	0.3 c.c. + 0.3 c.c. (1:10)	= +
0.3 c.c. + 0.3 c.c. (1:20)	= —	0.3 c.c. + 0.3 c.c. (1:20)	= —

It is seen in the above protocols that conditions similar to those observed in the experiments previously described had become established, and that the mutual precipitation of the sera, one by the other, was roughly proportionate to the amounts of antigen and precipitin contained in them.

Serum C, which contained, as we have seen, a considerable amount of antigen and very little precipitin, was noticed to have become distinctly turbid after forty-eight hours in the ice chest, and after seventy-two hours this turbidity had increased and a very fine and slight sediment had formed along the side of the glass. Morphological and cultural examination, moreover, showed this serum to be sterile.

Since the amount of serum C contained in the tube in which this turbidity appeared was considerable (eight cubic centimeters), centrifugation yielded a quantity of precipitate which was sufficient, after washing, to render two cubic centimeters of salt solution moderately turbid. This precipitate was then examined for its complement-fixing power and was found to fix completely 0.03 of a cubic centimeter of guinea pig complement, a quantity which caused hemolysis in the control tubes in ten minutes.

In this case, then, we obtained a rapid spontaneous precipitation of a serum containing both antigen and precipitin, more rapid than

any we have ever observed in normal serum. And the precipitate which resulted, after being washed free of serum, possessed distinct complement-fixing properties, a fact which would seem to justify us in assuming that, like other precipitates, it had resulted from the specific union of antigen and precipitin.

In considering the theories that have been advanced to explain these occurrences, the conception of mass action as accounting for the simultaneous presence of the two reacting bodies in the same serum seems entirely incompatible with the observation made both by Gay and Rusk and by us, that these sera do not of themselves fix alexin. Were the conception of the manner of union of these two reagents according to the laws of mass action representative of the true state of affairs, it would be necessary to assume the presence, in such sera, not only of the two reacting bodies free and dissociated, but also of a definite quantity of the united complex of the two, a state of equilibrium being established. If this were the case, the sera should, in agreement with all experience on the phenomenon of complement fixation, exert definite complement-binding power. Moreover, it has not been experimentally shown that colloidal substances—and we have reason to assume that both antigen and precipitin are of colloidal nature—react in accordance with the laws of mass action as observed for simpler chemical substances.

In regard to the opinion of von Dungern, this seems incompatible with the late spontaneous precipitation of such sera, which seems, both from the experiments of Merkel and from our own, to depend upon a slow and delayed eventual union between antigen and precipitin. Moreover, we believe that the explanation of the phenomena which we have to offer renders unnecessary many of the rather complicated premises of multiplicity advanced by von Dungern.

It has seemed to us that all the facts observed in connection with the problem above outlined are most simply explicable on the basis of colloidal protective action.

It is well known that the presence of small quantities of such colloids as gelatin will often prevent the precipitation of other colloids by electrolytes. Also the mutual precipitation of two colloids may occasionally be prevented by the presence of definite quantities of a third. The presence of a protective colloid would explain the

failure of union of the antigen and the precipitin in the sera under consideration and their lack of complement-fixing property. That serum components may under certain conditions possess such protective functions is clear from the work of Porges (10) regarding the protective action of heated serum, to which further reference will be made below. Furthermore, to conceive the state of affairs in the sera in this way would be compatible with the late spontaneous precipitation of the sera, an eventual failure of the protective action which may easily take place in colloid mixtures under the influence of slight evaporation, or even without this. That originally stable colloids may precipitate on standing is well known, and Bechold and Ziegler (9) state that suspensions of certain therapeutically useful colloids (camphor, etc.), which they attempted to hold in suspension for prolonged periods, for practical purposes, by the use of protective colloids, always eventually flaked out.

A phenomenon which is not so easily made compatible with the conception of a protective colloid is that of the mutual precipitation of two such sera when mixed. On further study, however, this feature of the experiments has become one of the strongest of the reasons which persuade us that our explanation is a very likely one.

In seeking analogy for this serum phenomenon with the various colloidal suspensions, the problem consisted in protecting two mutually precipitating colloids by a third, and this in such proportions, that the mixing of two such protected suspensions, each containing all three of the elements, would be followed by precipitation.

After considerable random experimentation we were partially successful by using arsenic trisulphide, with heated and unheated dog serum, following the observation of Porges (10), that native serum may precipitate mastic emulsions, while the same serum heated may exert an antiprecipitating or protective action.

We found that within certain limits of relative concentrations this was true of the action of heated and unheated dog serum both upon mastic and upon arsenic suspensions. By adding very small quantities of heated dog serum to these suspensions, the arsenic compound was precipitated. Slightly greater quantities of the heated serum again dispersed the suspension which became clear, though never as entirely clear as at first. The subsequent addition

of even considerable quantities of unheated dog serum to such mixtures did not lead to precipitation, although similar amounts of the unheated serum added to the same amounts of arsenic suspension invariably caused heavy precipitation. Great excesses of the unheated serum eventually had a similar dispersing action, but within a very wide zone of relative concentrations, the heated dog serum in comparatively small quantities protected the arsenic trisulphide from precipitation by the unheated serum. By varying relative amounts of the three ingredients, a number of such protected suspensions were obtained which on mixing were so changed that the ratio of the protective colloid to the other two was no longer wholly protecting and partial precipitation resulted. The details of these experiments are not given since they served merely to emphasize the probable validity of our reasoning.

A better method of simulating the behavior of the sera was obtained by the use of gum arabic, gelatin, and arsenic trisulphide. Thin emulsions of gelatin will precipitate arsenic trisulphide suspensions. Small amounts of gum arabic will act as a protective agent, preventing the precipitations.

The amount of the protecting substance necessary to prevent precipitation in any one mixture varies apparently with every change in the relative proportions of the two mutually precipitating colloids. Thus a considerable number of mixtures of the three can be made which will remain stable for days, the actual and relative quantities of the three ingredients differing in each of the mixtures. When two such mixtures are poured together, in many cases precipitation will result, varying in speed and completeness according to the particular quantitative relationship arrived at in the mixture.

An example of such an experiment follows:

Two solutions of colloidal arsenic sulphide were prepared, one containing 1 gm. per liter, the other containing 5 gm. per liter. With Kahlbaum's *Golddruck* gelatin, a solution was prepared containing 1 gm. per liter. A solution of gum arabic was prepared which contained 10 gm. per liter, this being made stronger than the gelatin solution to avoid too great dilution in the final mixtures. The gelatin solution was prepared twenty-four hours before being used, as freshly prepared gelatin has but slight precipitating power for arsenic sulphide, this power appearing to increase greatly with the aging of the solution.

For the purpose of demonstrating this analogy two protected solutions were prepared as follows:

Solution 1.—This consisted of 2 drops of gum arabic, 2 c.c. of gelatin, and 5 c.c. of the weaker arsenic solution.

Solution 2.—This consisted of 10 drops of gum arabic, 1 c.c. of gelatin, and about 4 c.c. of the stronger arsenic solution.

In each case the arsenic sulphide was added until there were signs of increasing opalescence or turbidity, this being done in order that the two solutions should each be as little overprotected as possible.

Portions of the two solutions were then mixed in equal proportions. In the course of a few minutes the mixture was noticeably more turbid than either of the original solutions. This turbidity continued to increase quite rapidly, and on the following morning after about sixteen hours of standing, the mixture was found to be completely flocculated out, while the original protected mixtures remained unprecipitated and showed about the same degree of opalescence as on the preceding night. The same condition of affairs was found to have persisted after five days. On the fifth day the less concentrated of the clear protected suspension began to settle out, and was completely precipitated within twenty-four hours. The other remained clear for four days more, but on the ninth day it began to precipitate slightly, the precipitation remaining incomplete.

This experiment was repeated several times, varying somewhat the relative amounts of the three components in the protected solutions, and precipitation was invariably found in the mixed solutions. In several cases a partial precipitation in one of the original protected solutions was also observed after twenty-four hours, this being wholly analogous to the occasional slow precipitation in the serum of an individual rabbit.

In these cases it appears, therefore, that a complete analogy to the observed conditions of the serum reactions has been found, and that all data observed in connection with sera in which antigen and precipitin are found side by side without reacting can be most simply explained on the conception of protective colloid action. Moreover, the chemical nature of the substances involved seems to add weight to our point of view.

It may even be that the presence of a protective colloid may, by inhibiting the union of antigen and precipitin within the body, protect the animal from intoxication during the early stages of immun-

ization when antigen and antibody are present simultaneously for longer or shorter periods. Were union between the two possible at such times in the circulation, an assumption necessitated both in the hypotheses of mass action and of multiplicity of precipitins, there would probably be an absorption of complement by these complexes, with, as shown by Friedberger, a consequent formation of powerful toxic products.

The fact, moreover, that mere heating will change the precipitating action, which certain sera have on inorganic colloids, to a protective one, seems to show that this latter function may justly be associated with delicate physical or chemical alterations of animal sera.

Furthermore, this point of view is strengthened by the fact that the mutual precipitation of sera such as those described takes place slowly, as does the mutual precipitation of two protected colloidal mixtures, in contradistinction to the more rapid precipitation which takes place when any of these sera is added to an antigen dilution, where the element of protection may be assumed to be practically eliminated by more extensively changed quantitative relations.

And knowing that but slight variation in the concentration of any one of the ingredients of any protected suspension may lead to union and precipitation, it is not unlikely that the harmful effects following suddenly upon the injection of considerable amounts of antigen into partially immunized animals may be explicable upon this basis. Thus the sudden addition of considerable amounts of antigen to the balanced suspension in the circulation may so disturb this balance that union between antigen and antibody can take place, the complex becoming sensitive to the alexin with consequent intoxication or shock, as in anaphylaxis.

BIBLIOGRAPHY.

1. Linossier, G., and Lemoine, G. H., *Compt. rend. Soc. de biol.*, 1902, liv. 85.
2. Eisenberg, P., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1903, xxxiv, 259.
3. Ascoli, M., *München. med. Wchnschr.*, 1902, xlix, 1409.
4. von Dungern, *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1903, xxxiv, 355.
5. Gay, F. P., and Rusk, G. Y., *University of California Publications in Pathology*, 1912, ii, 59, 73.

6. Fornet, W., and Müller, M., *Ztschr. f. biol. Tech. u. Method.*, 1908–9, i, 201; cited by Bonhoff, H., and Tsuzuki, M., *Ztschr. f. Immunitätsforsch., Orig.*, 1909–10, iv, 180.
7. Uhlenhuth, P., and Weidanz, O., Praktische Anleitung zur Ausführung des biologischen Eiweissdifferenzierungsverfahrens, Jena, 1909, 221.
8. Merkel, cited by Uhlenhuth, P., and Weidanz, O., *loc. cit.*, p. 228.
9. Bechhold, H., Die Kolloide in der Biologie und Medizin, Dresden, 1912, 65. Bechhold and Ziegler, cited by Bechhold, H., *loc. cit.*, p. 66.
10. Porges, O., in Kraus, R., and Levaditi, C., Handbuch der Technik und Methodik der Immunitätsforschung, Jena, 1909, ii, 1146.

THE RELATION OF THE LEUCOCYTIC BACTERIOLYSIN TO BODY FLUIDS.*

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The discovery that certain cells of the body are capable of giving off bactericidal substances has given rise to the hope that eventually cellular derivatives may prove of therapeutic value. It has been conceived, for example, that an efficient antiseptic may thus be obtained, which can be safely injected into body cavities and tissue spaces.

The bactericidal properties of many cellular products[1] have therefore been studied, particularly those of leucocytic products.[2] The leucocytic bacteriolysin has usually been tested, either dissolved in distilled water, in physiological saline, or in culture media. From observations in a previous paper,[3] I was led to test its properties when placed under conditions more nearly approaching those in the animal body. Its action was therefore tested when mixed with various normal and pathological body fluids and tissue derivatives.

The influence of a number of foreign substances on this bacteriolysin has already been studied. Of particular interest in the present connection is the observation of Pettersson,[4] that the bactericidal power is diminished in the presence of certain colloids. Pettersson[5] and others have shown that the leucocytic and the serum bacteriolysins are distinct substances. Kling and others, however, have observed an activation of an inactive leucocytic extract by the addi-

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[1] Conradi, H., *Beitr. z. chem. Phys. u. Path.*, 1902, i, 193.

[2] For a summary of previous work with leucocytic extracts, see Kling, C. A., *Ztschr. f. Immunitätsforsch.*, 1910, vii, 1. A review of the literature is also given by Schneider, R., *Arch. f. Hyg.*, 1909, lxx, 40.

[3] Manwaring, W. H., *Jour. Exper. Med.*, 1912, xvi, 249.

[4] Pettersson, A., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1911, lx, 286.

[5] Pettersson, A., *Ztschr. f. Immunitätsforsch.*, 1908–9, i, 52.

tion of serum. Weil, Schneider, and others have described the opposite effect, a diminution in bacteriolysis, as a result of serum addition.[6] Schattenfroh[7] found that the bactericidal power of an extract of rabbit leucocytes is independent of its salt content. Schneider[8] found that the addition of alkali diminishes the bactericidal power of the same extract.

MATERIAL AND TECHNIQUE.

The bacteriolysin studied in the present paper was obtained from horse leucocytes, according to the technique described in the previous communication. This extract had been heated to 57° C. for thirty minutes, had been dialyzed free from diffusible products, and then evaporated to dryness *in vacuo* at 45° C. It was tested after having been stored in a vacuum at 4° C. for about six months. The tests were made at room temperature.

EFFECTS OF SERUM ON THE BACTERIOLYSIN.

Inactive Serum.—The addition of a trace of inactive[9] homologous or foreign serum to an amount of leucocytic extract barely sufficient

TABLE I.

Effect of Inactive Serum on the Bacteriolysin.

The fluids to be tested were made up to equal volumes (1 c.c.) by the addition of ¼ physiological saline solution (0.22 per cent. sodium chloride). Each fluid was then inoculated with a loopful of an eighteen hour broth culture of *B. typhosus*. Plates were made from the resulting mixtures at the times indicated. The table records the number of colonies on the plates thus obtained. The serum tested in this table was horse serum that had spontaneously become inactive by long standing in the ice chest.

Material tested.	Time of plating.				
	1 min.	1 hr.	2 hrs.	5 hrs.	24 hrs.
Bacteriolysin alone	950	90	10	8	650
Bacteriolysin + 0.5% inactive serum	510	7	0	0	15
50% inactive serum alone	1,000	960	1,010	1,220	16,000

[6] Kling, C. A., *loc. cit.*, p. 30.

[7] Schattenfroh, A., *Arch. f. Hyg.*, 1899, xxxv, 135.

[8] Schneider, R., *loc. cit.*, p. 120.

[9] Serum was usually inactivated, either by heating it to 57° C. for 30 min., or by allowing it to stand at room temperature till its bactericidal properties had disappeared.

for complete sterilization usually increases slightly the rapidity and the completeness of its bacteriolytic action (table I). It is possible that a more pronounced phenomenon of this nature is the phenomenon described by Kling and others as an activation of the extract.

The addition of larger amounts of inactive serum invariably leads to a more or less complete inhibition of the bactericidal properties (table II). Sera differ considerably in the amount of this antibactericidal action, rabbit serum, for example, having approximately four times the antibactericidal power of horse serum.

TABLE II.

Effect of Inactive Serum on the Bacteriolysin.

The tests were made as in table I, but with larger amounts of the inactive serum. Test organism, *B. typhosus*. Test fluids contained 0.22 per cent. sodium chloride.

Material tested.	Time of plating.				
	1 min.	1 hr.	2 hrs.	5 hrs.	24 hrs.
Bacteriolysin alone	600	6	0	0	0
Bacteriolysin + 1.5% inactive serum	600	5	0	0	0
Bacteriolysin + 3% inactive serum	800	20	2	0	30
Bacteriolysin + 6% inactive serum	850	50	10	0	2,000
Bacteriolysin + 12% inactive serum	850	400	250	200	3,000
Bacteriolysin + 25% inactive serum	950	550	500	400	4,000
Bacteriolysin + 50% inactive serum	1,020	850	820	800	16,000
100% inactive serum alone	1,050	1,000	950	1,020	20,000

Active Serum.—The effects of the addition of active serum are less easily determined, due to the bactericidal properties of the serum itself. By carefully adjusting the relative amounts of serum and extract, however, it is possible to show (table III) that active serum also is capable of inhibiting or destroying the bacteriolytic action. But what from the practical point of view is possibly of equal importance is the fact that the active serum as well as the extract loses its bactericidal power, as a result of such admixture. This gives the phenomenon of two bactericidal substances, active serum and active leucocytic extract, added to each other to produce a fairly good culture medium for bacteria.

TABLE III.

Effect of Active Serum on the Bacteriolysin.

Guinea pig serum was selected on account of its comparatively weak bactericidal action on *B. typhosus*. Tests as in table I. Test fluids contained 0.22 per cent. sodium chloride.

Material tested.	Time of plating.			
	1 min.	1 hr.	3½ hrs.	24 hrs.
10% guinea pig serum alone	1,300	450	0	0
Bacteriolysin alone	450	5	0	0
Bacteriolysin+10 % guinea pig serum	1,200	1,000	750	15,000

EFFECT OF NORMAL TISSUE FLUIDS ON THE BACTERIOLYSIN.

Cerebrospinal fluid was selected as the easiest obtainable normal tissue fluid. The addition of cerebrospinal fluid to leucocytic extract produces nearly as great an inhibition or destruction of its bacteriolytic properties as the addition of serum itself (table IV).

TABLE IV.

Effect of Cerebrospinal Fluid on the Bacteriolysin.

Human cerebrospinal fluid (apparently normal) obtained by lumbar puncture for diagnostic purposes was tested as in table II. Test organism, *B. typhosus*. Dilutions made with 0.22 per cent. sodium chloride. A similar action was obtained with the cerebrospinal fluid of the monkey and the dog.

Material tested.	Time of plating.			
	1 min.	1 hr.	4 hrs.	24 hrs.
Bacteriolysin alone	300	1	0	0
Bacteriolysin+ 2% cerebrospinal fluid	350	0	0	0
Bacteriolysin+ 4% cerebrospinal fluid	700	20	1	0
Bacteriolysin+ 8% cerebrospinal fluid	750	250	4	3,000
Bacteriolysin+16% cerebrospinal fluid	800	300	250	10,000
Bacteriolysin+33% cerebrospinal fluid	1,000	600	700	15,000
Bacteriolysin+66% cerebrospinal fluid	1,000	750	800	25,000
100 % cerebrospinal fluid alone	1,000	1,000	1,200	20,000

EFFECTS OF PATHOLOGICAL FLUIDS ON THE BACTERIOLYSIN.

Pathological Effusion.—The action of a pathological effusion is also practically the same as that of serum (table V).

TABLE V.

Effect of Pathological Effusion on the Bacteriolysin.

The effusion here tested was obtained by injecting aleuronat into the pleural cavity of a dog. The resulting pleural exudate was aspirated, twenty-four hours later, was centrifuged free from cellular elements, and then inactivated by heating it to 57° C., for twenty minutes. Tests of its antibactericidal action were made as in table II. Test organism, *B. typhosus*. Test fluids contained 0.22 per cent. sodium chloride.

Material tested.	Time of plating			
	1 min.	45 min.	3½ hrs.	24 hrs.
Bacteriolysin alone	300	0	0	0
Bacteriolysin + 1.5% pleural effusion	450	3	0	0
Bacteriolysin + 3% pleural effusion	500	20	0	0
Bacteriolysin + 6% pleural effusion	650	50	1	10
Bacteriolysin + 12% pleural effusion	800	60	2	40
Bacteriolysin + 25% pleural effusion	1,000	500	250	6,000
Bacteriolysin + 50 % pleural effusion	1,150	1,000	650	20,000
50% pleural effusion alone	1,100	1,100	900	2,000

Autolytic Products.—Substances given off during the aseptic autolysis of tissues are also strongly antibactericidal (table VI), as are also most of the products obtained by the bacterial decomposi-

TABLE VI.

Effect of Autolytic Products on the Bacteriolysin.

The kidney of a dog was removed with aseptic precautions, ground up in sterile sand, and the resulting finely divided tissue was washed three times by centrifugation with physiological saline solution. The serum-free tissue fragments were now suspended in three volumes of ¼ physiological saline solution, and the resulting suspension was incubated over night. The suspension was then freed from tissue fragments by centrifugation and its antibactericidal powers were tested. Test organism, *B. typhosus*. Test fluids contained 0.22 per cent. sodium chloride. A similar antibactericidal action was obtained with the autolytic products of liver, spleen, and heart muscle.

Material tested.	Time of plating.			
	1 min.	1 hr.	4 hrs.	24 hrs.
Bacteriolysin alone	600	20	0	0
Bacteriolysin + 0.75% autolytic products	850	100	0	0
Bacteriolysin + 1.5% autolytic products	850	150	6	0
Bacteriolysin + 3% autolytic products	850	350	150	25
Bacteriolysin + 6% autolytic products	900	500	350	600
Bacteriolysin + 12% autolytic products	950	600	400	2,000
Bacteriolysin + 25% autolytic products	950	700	600	15,000
Bacteriolysin + 50% autolytic products	980	850	850	30,000
50% autolytic products, alone	1,100	1,050	950	40,000

tion of tissues. None of the autolytic products thus far obtained have been in themselves bactericidal. A number of them have had the power of inhibiting bacterial multiplication, the property studied by Conradi.[10]

Of particular interest are the effects of products obtained by the prolonged autolysis of leucocytes themselves. On prolonged autolysis, both homologous leucocytes and foreign leucocytes give off strongly antibactericidal substances (table VII). The formation or liberation of these antibactericidal leucocytic products undoubtedly explains the fact, noted in the previous paper, that, in order to obtain an active leucocytic extract, the extraction process must be interrupted at a certain stage. Too prolonged an extraction usually gives an inactive extract.

TABLE VII.

Effect of the Products of the Prolonged Autolysis of Leucocytes on the Bacteriolysin.

Horse leucocytes were extracted at 37° C. for two hours in ¼ physiological saline solution, and were then freed from the supernatant fluid by centrifugation. The partially extracted leucocytes were now suspended in three volumes of ¼ physiological saline solution and the autolysis was allowed to continue at 37° C. over night. The resulting autolytic products were then freed from cellular elements and tested for their antibactericidal action. Test organism, *B. typhosus*. Test fluids contained 0.22 per cent. sodium chloride.

Material tested.	Time of plating.			
	1 min.	45 min.	3½ hrs.	24 hrs.
Bacteriolysin alone	600	50	0	0
Bacteriolysin + 0.5% leucocytic products	750	60	0	0
Bacteriolysin + 1% leucocytic products	800	50	0	0
Bacteriolysin + 2% leucocytic products	800	150	0	5
Bacteriolysin + 4% leucocytic products	800	600	200	15
Bacteriolysin + 8% leucocytic products	750	700	350	300
Bacteriolysin + 16% leucocytic products	800	600	400	500
Bacteriolysin + 33% leucocytic products	950	850	500	1,000
Bacteriolysin + 66% leucocytic products	950	850	750	2,000
66 % leucocytic products alone	850	750	800	2,000

QUANTITATIVE RELATIONS.

Amount.—The relation between the amount of bacteriolysin tested and the amount of serum or other body fluids necessary to

[10] Conradi, H., *loc. cit.*, p. 222.

produce a given decrease in its bactericidal power is indicated in table VIII.

From this table it is seen that the amount of serum necessary to produce a given change in the bactericidal power increases with the amount of the bacteriolysin tested.

TABLE VIII.

Quantitative Relations.

Parallel tests of the antibactericidal power of horse serum, with three different amounts of the bacteriolysin. Test organism, *B. typhosus*. Test fluids contained 0.22 per cent. sodium chloride.

Material tested.	Serum necessary to reduce bacteriolysis.				Average.
	Slightly.	Markedly.	Nearly completely.	Completely.	
0.25 c.c. bacteriolysin.....	3%	5%	7%	11%	6.5%
0.35 c.c. bacteriolysin.....	7%	11%	16%	22%	14.0%
0.50 c.c. bacteriolysin.....	7%	16%	22%	33%	19.5%

Rate.—The neutralization, destruction, or binding of the bactericidal substance takes place instantaneously on the addition of the serum or body fluid. The reaction differs in this particular from a number of serum reactions, which require a certain length of time before becoming complete. Duplicate mixtures of serum and bacteriolysin tested immediately and after standing for various lengths of time show, within the limits of the experimental error, the same bactericidal powers.

ANTIBACTERICIDAL ACTION ANALYZED.

An effort was made to determine which of the components of serum and body fluids are responsible for the antibactericidal action.

Serum Colloids.—Serum colloids were obtained by dialyzing serum free from diffusible products. The precipitated globulins were put in solution by the addition of a minimum known amount of sodium chloride, and in testing the antibactericidal action the same amount of sodium chloride was used in all parallel and control tubes. The results of the analysis are shown in table IX. From this table it appears that about half the antibactericidal action of serum is due to the serum colloids.

TABLE IX.

Effect of the Serum Colloids on the Bacteriolysin.

The antibactericidal action of inactive horse serum was compared with that of the colloids obtained from the same serum. Test organism, *B. typhosus.* Test fluids contained 0.22 per cent. sodium chloride.

Material tested.	Time of plating.			
	1 min.	1 hr.	4 hrs.	24 hrs.
Bacteriolysin alone	950	60	0	0
Bacteriolysin + 2% whole serum	950	50	0	0
Bacteriolysin + 4% whole serum	700	0	0	0
Bacteriolysin + 8.5% whole serum	600	6	0	50
Bacteriolysin + 17% whole serum	800	350	70	8,000
Bacteriolysin + 33% whole serum	1,100	1,000	800	10,000
Bacteriolysin + 66% whole serum	1,150	1,000	1,000	30,000
Bacteriolysin + 2% serum colloids	900	2	0	0
Bacteriolysin + 4% serum colloids	700	15	0	0
Bacteriolysin + 8.5% serum colloids	600	1	0	0
Bacteriolysin + 17% serum colloids	600	30	0	60
Bacteriolysin + 33% serum colloids	700	400	100	1,200
Bacteriolysin + 66% serum colloids	1,050	650	750	2,000
66% whole serum alone	1,100	1,000	1,050	25,000
66% serum colloids alone	1,200	1,100	1,100	20,000

Serum Crystalloids.—The diffusible serum components were obtained by collecting the water from the dialysis above, and evaporating it to a small volume. The action of these diffusible products is shown in table X. This table confirms the previous finding, since

TABLE X.

Effect of the Serum Crystalloids on the Bacteriolysin.

The serum crystalloids, separated by dialysis from the serum colloids tested in table IX, were tested for their antibactericidal action. Test organism, *B. typhosus.* Dilutions made with 0.2 per cent. sodium chloride.

Material tested.	Time of plating.			
	1 min.	1 hr.	4 hrs.	24 hrs.
Bacteriolysin alone	950	60	0	0
Bacteriolysin + 2% serum crystalloids	600	4	0	0
Bacteriolysin + 4% serum crystalloids	1,000	150	10	0
Bacteriolysin + 7.5% serum crystalloids	1,000	100	2	0
Bacteriolysin + 15% serum crystalloids	600	50	0	2
Bacteriolysin + 30% serum crystalloids	800	120	30	3,000
Bacteriolysin + 60% serum crystalloids	950	700	700	30,000
60% serum crystalloids alone	1,100	1,050	1,100	20,000

it shows that approximately half the antibactericidal action of serum is due to its non-colloidal or diffusible components. The fact that the diffusible serum components possess a considerable antibactericidal action agrees with the observation that cerebrospinal fluid is strongly antibactericidal. Normal cerebrospinal fluid contains only a trace of colloidal (proteid) material, but is rich in diffusible serum components.[11]

Ringer Solution.—The action of Ringer solution is similar to the action of the diffusible serum products (table XI).

TABLE XI.

Influence of Ringer Solution on the Bacteriolysin.

Equal amounts of the bacteriolysin were dissolved in distilled water, half strength Ringer solution, and full strength Ringer solution, and then tested for their bactericidal power. The Ringer solution used in these tests had the following composition: 2.5 c.c. *m/1* $NaHCO_3$ + 2 c.c. *m/1* $CaCl_2$ + 4 c.c. *m/1* KCl + 9 gm. NaCl + 990 c.c. H_2O. Test organism, *B. typhosus.*

Material tested.	Time of plating.			
	1 min.	1 hr.	4 hrs.	24 hrs.
Bacteriolysin in distilled water	500	30	0	0
Bacteriolysin in 50% Ringer solution	800	150	20	15,000
Bacteriolysin in 100% Ringer solution	950	600	500	40,000

Sodium Chloride.—It was pointed out in the previous paper that sodium chloride is antibactericidal for leucocytic extract.[12] Its antibactericidal action, however, is distinctly less than that of

[11] For analyses of normal and pathological cerebrospinal fluids, see Mott, F. W., *Lancet,* 1910, ii, 1, 79.

[12] The fact that an extract of horse leucocytes is decreased in its bactericidal power by the addition of sodium chloride does not agree with Schattenfroh's original observation (*loc. cit.*) that the action of the leucocytic bacteriolysin is independent of the salt content of the fluid tested. Schattenfroh's observation is of considerable importance, because it was cited as evidence that the leucocytic bacteriolysin and the serum bacteriolysin are two distinct substances, the bactericidal properties of serum being influenced by variations in its salt content.

The fact that an extract from horse leucocytes changes its bactericidal power on altering the amount of sodium chloride it contains does not, however, furnish evidence of the identity of the serum and leucocytic bacteriolysins. The serum bacteriolysin is active in the presence of sodium chloride, but inactive in distilled water. The leucocytic bacteriolysin is active in distilled water, but almost

Ringer solution (table XII). The antibactericidal action of sodium chloride is not due to a destruction or permanent injury of the bacteriolysin, since on removing the sodium chloride by dialysis the bactericidal power is restored quantitatively.

TABLE XII.

Comparative Antibactericidal Action of Physiological Saline Solution and Ringer Solution.

Equal amounts of the bacteriolysin were dissolved in distilled water, physiological saline solution (0.9 per cent. sodium chloride), and Ringer solution, and then tested for their bactericidal power. Test organism, *B. typhosus.*

Material tested.	Time of plating.			
	1 min.	1 hr.	4 hrs.	24 hrs.
Bacteriolysin in distilled water	1,000	350	0	0
Bacteriolysin in physiological saline solution	1,000	600	400	1,500
Bacteriolysin in Ringer solution	1,000	900	900	5,000

Alkalies.—The addition of alkali to the bacteriolysin causes a rapid decrease in its bactericidal power (table XIII). The bacteriolytic action, for example, is nearly completely abolished in the

TABLE XIII.

Influence of Alkali on the Bacteriolysin.

Equal amounts of the bacteriolysin were tested alone and in the presence of increasing amounts of sodium hydroxide. Test organism, *B. typhosus.* Test fluids contained 0.22 per cent. sodium chloride.

Material tested.	Time of plating.			
	1 min.	1½ hrs.	4 hrs.	24 hrs.
Bacteriolysin alone	650	100	2	10
Bacteriolysin + 0.0004% sodium hydroxide	800	200	20	50
Bacteriolysin + 0.0008% sodium hydroxide	760	250	30	70
Bacteriolysin + 0.0016% sodium hydroxide	860	550	300	350
Bacteriolysin + 0.0033% sodium hydroxide	780	500	600	550
Bacteriolysin + 0.0067% sodium hydroxide	750	520	500	400
0.0067% sodium hydroxide alone	770	600	600	650

inactive in the presence of sodium chloride. The observation therefore tends to strengthen Schattenfroh's conclusion that the two lysins are distinct. It is probable that the differences in the results are due to differences in the methods of preparing the leucocytic extracts.

presence of 0.003 per cent. sodium hydrate. A similar, though possibly slightly less pronounced inhibition is produced by sodium carbonate. The antibactericidal action of alkalies is not due to a destruction or permanent injury of the bactericidal agent, since on neutralizing an inactive mixture of bacteriolysin and alkali the bactericidal power is restored quantitatively.

Acids.—The determination of the effects of acids on the bacteriolysin is limited by the toxicity of most acids. Hydrochloric acid, within the limits of its toxicity, apparently has no effect. The range of the test is somewhat greater with acetic acid (table XIV), but within the limits of the experimental error, acetic acid also is without a distinct action on the leucocytic bacteriolysin.

TABLE XIV.

Influence of Acetic Acid on the Bacteriolysin.

Equal amounts of the bacteriolysin were tested alone and in the presence of increasing amounts of acetic acid. Test organism, *B. typhosus*. Test fluids contained 0.22 per cent. sodium chloride.

Material tested.	Time of plating.			
	1 min.	1½ hrs.	4 hrs.	24 hrs.
Bacteriolysin alone	650	100	2	7
Bacteriolysin + 0.0003% acetic acid	750	90	2	8
Bacteriolysin + 0.0006% acetic acid	800	100	4	50
Bacteriolysin + 0.0012% acetic acid	900	90	2	30
Bacteriolysin + 0.0025% acetic acid	700	60	6	0
Bacteriolysin + 0.0050% acetic acid	650	60	3	0
0.0050% acetic acid alone	1,000	850	750	700

Boric acid is less toxic for bacteria than acetic acid, and can be tested in much higher concentrations (table XV). When added to leucocytic extract in small amounts, boric acid also is without any distinct action on bacteriolysis. It is only when the amount reaches 0.2 per cent. that a slight slowing in the rate of the bacteriolysis is observed. It is questionable, however, whether this is really due to acid action, or may not result from the necessary changes in osmotic condition. With 1.25 per cent. boric acid, bacteriolysis is distinctly reduced.

TABLE XV.

Influence of Boric Acid on the Bacteriolysin.

Equal amounts of the bacteriolysin were tested alone and in the presence of increasing amounts of boric acid. Test organism, *B. typhosus*. Test fluids contained 0.22 per cent. sodium chloride.

Material tested.	Time of plating.			
	1 min.	1½ hrs.	4 hrs.	24 hrs.
Bacteriolysin alone	550	30	0	0
Bacteriolysin + 0.025% boric acid	450	1	0	0
Bacteriolysin + 0.05% boric acid	700	5	0	0
Bacteriolysin + 0.1% boric acid	800	40	0	0
Bacteriolysin + 0.2% boric acid	800	300	6	0
Bacteriolysin + 0.4% boric acid	750	300	3	0
Bacteriolysin + 0.8% boric acid	750	300	0	0
Bacteriolysin + 1.25% boric acid	750	400	100	20
Bacteriolysin + 2.5% boric acid	800	300	200	50
2.5% boric acid alone	750	750	700	650

Quantitative Relations.—The relative amount of the destruction or inhibition of bacteriolysis that is due to each of the above components has not been determined. With serum, however, it is probable that about half the antibactericidal action is due to the serum colloids, about a quarter to the neutral diffusible products, and a quarter to the diffusible alkalies.

MECHANISM OF THE ANTIBACTERICIDAL ACTION.

The manner in which the various substances above enumerated antagonize or overcome the bactericidal action has not been determined. Two methods are theoretically possible. First, the antibactericidal substances may enter into a direct chemical relation with the bacteriolysin, with the formation of non-bactericidal combination products or split products. Or second, the antibactericidal substance may enter into direct chemical relation with the bacteria, making them in some way insusceptible to the action of the bacteriolysin. This second method might conceivably be somewhat similar to the antagonistic salt actions studied by Loeb[13] and his co-workers, in which one chemical substance so alters the permeability of the limiting cell membrane as to prevent the entrance into the cell of the toxic agent. It is quite possible, and even probable,

[13] Loeb, J., The Dynamics of Living Matter, New York, 1906, 70.

that the antagonistic action of any one of the antibactericidal body fluids is a combination of a direct action of certain components on the bacteriolysin and of other components on the bacteria.

METHODS TO PREVENT THE ANTIBACTERICIDAL ACTION.

Quantity.—An attempt was made to devise a method to overcome or prevent the antibactericidal action of serum and body fluids. The first method that suggested itself was to exhaust the antibactericidal power of the inhibiting fluid by increasing the amount of bacteriolysin added to it. It is possible, under certain conditions, to overcome the antibactericidal action of sodium chloride by this means (table XVI).

TABLE XVI.

Attempt to Exhaust the Antibactericidal Action of a Neutral Salt.

Parallel tests with multiple doses of the leucocytic bacteriolysin dissolved in physiological saline (0.9 per cent. sodium chloride). Test organism, *B. typhosus.*

Material tested.	Time of plating.			
	1 min.	1 hr.	4 hrs.	24 hrs.
Bacteriolysin in distilled water	650	40	5	0
Bacteriolysin in physiological saline	600	550	600	25,000
2 × bacteriolysin in physiological saline	600	350	300	20,000
4 × bacteriolysin in physiological saline	400	10	5	2,000
8 × bacteriolysin in physiological saline	400	5	0	0

It is also possible to overcome part of the antibactericidal action of Ringer solution by the same means (table XVII).

TABLE XVII.

Attempt to Exhaust the Antibactericidal Action of Ringer Solution.

Parallel tests with multiple doses of the leucocytic bacteriolysin dissolved in Ringer solution. Test organism, *B. typhosus.*

Material tested.	Time of plating.			
	1 min.	1 hr.	4 hrs.	24 hrs.
Bacteriolysin in distilled water	1,000	70	1	0
Bacteriolysin in Ringer solution	1,000	950	1,000	50,000
2 × bacteriolysin in Ringer solution	1,000	700	600	50,000
4 × bacteriolysin in Ringer solution	1,000	300	70	20,000
8 × bacteriolysin in Ringer solution	950	150	20	2,500

TABLE XVIII.

Attempt to Exhaust the Antibactericidal Action of Body Fluids.

Parallel tests with multiple doses of the leucocytic bacteriolysin dissolved in human cerebrospinal fluid. Test organism, *B. typhosus*. Dilutions made with 0.22 per cent. sodium chloride. A practically identical result was obtained with inactivated pathological transudate and with inactive serum.

Material tested.	Time of plating.			
	1 min.	1 hr.	4 hrs.	24 hrs.
Bacteriolysin in distilled water	1,100	350	0	0
Bacteriolysin in cerebrospinal fluid	1,100	1,000	1,000	6,000
2 × bacteriolysin in cerebrospinal fluid	1,050	1,000	1,000	8,000
4 × bacteriolysin in cerebrospinal fluid	1,050	950	1,000	8,000
8 × bacteriolysin in cerebrospinal fluid	1,100	1,000	1,000	6,000

Attempts to overcome the antibactericidal action of cerebrospinal fluid (table XVIII), of pathological transudates, and of serum by this means, however, have thus far been unsuccessful. Apparently no amount of leucocytic bacteriolysin added to these fluids, when tested in their full concentration, is able to exhaust their antibactericidal capacity. If the fluids, however, are diluted, their action is more nearly that of Ringer solution.

TABLE XIX.

Attempt to Diminish the Antibactericidal Action of Body Fluids.

Parallel samples of a non-bactericidal mixture of the leucocytic bacteriolysin and serum were tested alone and in the presence of increasing amounts of boric acid. Test organism, *B. typhosus*. Test fluids contained 0.22 per cent. sodium chloride. A practically identical result was obtained with a non-bactericidal mixture of the bacteriolysin and cerebrospinal fluid. A similar restoration of part of the original bactericidal power can be obtained with acetic acid.

Material tested.	Time of plating.			
	1 min.	1 hr.	4 hrs.	24 hrs.
Bacteriolysin alone	1,200	60	0	0
Bacteriolysin + serum	1,100	1,050	1,100	50,000
Bacteriolysin + serum + 0.017% boric acid	1,050	1,000	1,000	8,000
Bacteriolysin + serum + 0.035% boric acid	1,100	900	850	6,000
Bacteriolysin + serum + 0.07% boric acid	1,050	850	800	1,200
Bacteriolysin + serum + 0.15% boric acid	1,050	750	800	850
Bacteriolysin + serum + 0.3% boric acid	1,000	600	600	350
Bacteriolysin + serum + 0.6% boric acid	1,000	600	600	400
Bacteriolysin + serum + 1.25% boric acid	1,100	550	500	350
Bacteriolysin + serum + 2.5% boric acid	1,050	600	400	300

Acidulation.—A second method that suggested itself was to neutralize the antibactericidal alkalies. This method is partially successful. The addition, for example, of boric acids to a non-bactericidal mixture of the bacteriolysin and body fluids occasionally restores part of the original bactericidal power (table XIX). In no case thus far tested, however, has more than a small fraction of the original power been restored by this means. The result differs in this respect from the result reported by Lamar,[14] who was able to restore completely the inhibited bactericidal power of certain soaps by adding boric acid to an inactive mixture of the soap and serum.

SUMMARY.

1. An extract of horse leucocytes is strongly bactericidal when dissolved in distilled water; it has considerable bactericidal power when dissolved in physiological saline; but it loses its bactericidal properties when mixed with blood serum or with normal or pathological tissue fluids.

2. About half the antibactericidal action of blood serum is due to the serum colloids, about a quarter to the neutral serum crystalloids, and a quarter to the diffusible alkalies. Diffusible acids have no antibactericidal action.

3. The addition of boric acid to an inactive mixture of leucocytic extract and serum or other body fluid occasionally restores part of the original bactericidal power, but never more than a small fraction of that power.

[14] Lamar, R. V., *Jour. Exper. Med.*, 1911, xiii, 1, 380; xiv, 256.

THE REACTION OF THE LUNGS TO THE INTRABRONCHIAL INSUFFLATION OF KILLED VIRULENT PNEUMOCOCCI AND OF PLAIN STERILE BOUILLON.*

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In a foregoing communication[1] we reported that the intrabronchial insufflation of a non-virulent strain of pneumococcus produced a lesion which had the gross appearance of a lobar pneumonia. This fact led us to investigate the effects of insufflation of killed virulent pneumococci and of uninoculated sterile bouillon. Both subjects were studied in short series of experiments.

INSUFFLATION OF KILLED VIRULENT PNEUMOCOCCI.

The problem was investigated in a series of ten dogs. A virulent strain of pneumococcus was grown in broth for about twenty hours, and was then killed by heating to 60° C. for fifteen minutes. Control cultures were made and found to be sterile. Doses of fifteen cubic centimeters of killed pneumococci were introduced into the lungs of six dogs; they showed no signs of illness. Only in one animal was there a rise of temperature of 1° C.

One dog was killed twenty-four hours after inoculation. Its lungs showed no pleurisy, and all the lobes were well aerated except the right posterior, which was dark in its posterior third but neither heavy nor swollen. On section this portion of the lung was very moist; thin, frothy fluid ran from the bronchi, and there was a mottled appearance due to the presence of partly solid areas of small size. By an irregular line of congestion this engorged lung substance shaded into the completely normal portion. Microscopic examination showed the lesion to be one of edema and congestion, with some leucocytic exudate in small groups of alveoli. There was

* Received for publication, January 9, 1913.

[1] Wollstein, M., and Meltzer, S. J., *Jour. Exper. Med.*, 1913, xvii, 353.

complete absence of fibrin. Cultures from the heart's blood and lung remained sterile. Smears from the lung showed pneumococci in fair numbers. Evidently some of the injected broth containing the dead but still stainable pneumococci was mixed with the frothy fluid exuding from the bronchi. There was no phagocytosis of the pneumococci.

Two dogs were killed forty-eight hours after insufflation. The lungs showed a patchy congestion in the posterior right lobe besides a small, dark, semisolid area (one centimeter square) near the base of this lobe. Microscopically areas of intra-alveolar leucocytic exudation were found, but there was no fibrin in the alveolar contents. Cocci were still demonstrable in smears, but the cultures remained sterile.

In three dogs, which were killed after three days, the lungs were the seat of superficial areas of congestion in the posterior lobes, with no sign of hepatization. Microscopically there was evidence of phagocytosis of red cells and leucocytes by macrophages. The resolving area was a very small one and no hepatized lobules remained.

Four other dogs received doses of dead cocci enriched three times. Their lungs showed similar lesions of congestion, edema, and very small areas of mild inflammation, the lesions being no more extensive nor severe than those resulting from the smaller doses. A very small amount of fibrin was found in the alveolar exudate of a few lobules, polymorphonuclear leucocytes and small numbers of red cells being present as well. Other alveoli contained granular serum alone or with desquamated epithelium. The lesion bore no resemblance to that produced by living, virulent pneumococci in the same length of time.

These experiments then showed that the lesions produced by intrabronchial insufflation of killed cultures of virulent pneumococci, still containing stainable organisms, are not similar to the massive pneumonias produced by the insufflation of living pneumococci, but consist of inextensive, mild inflammation of a patchy and superficial character. Mild leucocytic exudation into the alveoli, which also contained a few epithelial cells and some serum, was confined to a few lobules. Fibrin was practically absent. The limited exudates were almost completely absorbed on the third day.

INSUFFLATION OF STERILE BROTH.

In the foregoing experiments the insufflated material contained, besides the broth, substances resulting from the breaking down of some of the pneumococci, and many still morphologically normal, stainable cocci. The question had still to be settled as to the nature of the reaction which the insufflation of sterile broth alone produces. Lamar and Meltzer[2] stated that insufflation of salt solution, broth, serum, etc., was not followed by consolidation. We give briefly some details of the observations made on a small series of animals receiving, by intrabronchial insufflation, uninoculated sterile bouillon of the kind generally used for cultivating pneumococci.[3]

The experiments were made on six dogs. Four of the animals received twenty cubic centimeters of broth each; to the other two fifteen cubic centimeters were given. The dogs did not appear to be ill after the insufflation. The larger dose was followed by a rise of temperature of 0.7° C. in two cases, and of 0.8° C. in one other instance; but only in the case of one dog did the temperature remain higher than normal during the day following inoculation.

The lungs of the dogs killed twenty-four hours after receiving the broth looked well aerated and pink. In the posterior lobes there were scattered areas of a darker color, giving the lungs a mottled appearance. These darker portions were elastic and aerated. On section they proved to be congested and very moist, exuding a thin, frothy fluid which was chiefly broth; in no case was there any sign of consolidation. The trachea, bronchi, and lymph nodes were normal in appearance. In one instance the posterior half of the right lung was found to be the seat of an extensive hemorrhage, but no inflammation was present. The pleura had retained its lustre perfectly.

Forty-eight hours after the administration of the broth the posterior lobes were still congested, but there was no evidence of consolidation. The dog whose temperature had remained 0.6° C. above normal showed very small subpleural hemorrhages over all the lobes of the right lung, and the congestion of the lung substance was intense. There was no edema and no hepatization.

[2] Lamar, R. V., and Meltzer, S. J., *Jour. Exper. Med.*, 1912, xv, 133.
[3] Lamar, R. V., and Meltzer, S. J., *loc. cit.*, p. 136.

On microscopic examination the capillaries in the alveolar walls as well as the larger blood vessels in the lung framework were found to be distended with red blood cells, of which small numbers had escaped into some of the alveoli. Granular material (serum or broth) was found in many alveoli, and there were several groups of air sacs which contained polymorphonuclear leucocytes. The bronchi and the interlobular septa were quite normal.

Cultures made from the lungs and heart's blood of these six animals remained sterile.

These observations show that sterile bouillon causes a pronounced congestion of the lung tissue with which it comes in contact and that exceptionally it may lead to slight hemorrhage. It brings about a negligible amount of inflammation, visible only on microscopic examination. The congestion may last forty-eight hours. It is possible that some of the bouillon remains unabsorbed for that length of time, thus serving as a continuous irritant.

Since all the animals in these two series of experiments had been killed with chloroform, it seemed advisable to examine the lungs of normal, untreated dogs, killed in the same manner. Such an examination showed that the lungs were pink and aerated, uniformly congested in the posterior lobes, and less markedly congested in the middle and anterior lobes. The trachea, bronchi, and lymph nodes were normal. There was no mottling of the lungs with darker patches, and the lungs contained much less blood than did those of the animals treated with bouillon. On microscopic examination the bronchi were all normal; the capillaries were distended with red cells, some of which were found in the alveoli, together with desquamated epithelium. There was no edema, and no leucocytes had left the blood vessels.

The killing with chloroform causes some degree of congestion in the lungs, more marked in both posterior lobes. However, this congestion is definitely less marked than that which follows the injection of broth and which is confined mostly to the posterior lobe of one lung. We may therefore be quite certain that the essential part of the congestion which was found to exist in one of the posterior lobes twenty-four or forty-eight hours after an intrabronchial insufflation of bouillon was due to the irritation of the bouillon present in that part of the lung and not to the chloroform.

SUMMARY.

The experiments show that intrabronchial insufflation of a culture of virulent pneumococcus killed by heat and still containing stainable organisms produces an inextensive, mild, patchy, super-

ficial inflammation of the lung tissue bearing no similarity to the lesions produced by the living pneumococcus, and that insufflation of sterile bouillon causes a pronounced congestion of the lung tissue with which it comes in contact, sometimes lasting for forty-eight hours.

THE INFLUENCE OF THE VAGUS NERVES ON THE FARADIZED AURICLES IN THE DOG'S HEART.*

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Plates 71 to 78.

The experiments on which this paper is based were undertaken in order to study the influence of the vagus nerves on auricular fibrillation. The problem was suggested by the study of a patient suffering from transient attacks of auricular fibrillation, whose heart was affected to an unusual degree by pressure over either of the vagi. The patient was of neurotic temperament, and the circumstances under which the attacks of auricular fibrillation occurred indicated that the controlling nervous mechanism of the heart might have played an important part in determining the onset and determination of the attacks. The case itself will be described in detail in another communication. The experiments were undertaken with the idea of determining whether the activity of the inhibitory mechanism played any part in rendering the heart susceptible to auricular fibrillation or in preventing its occurrence.

Certain experimental investigations have been carried on regarding the influence of the vagi on the fibrillating auricles. There has not been, however, entire agreement of interpretation of results by the various workers. Some have said that vagus stimulation may cause auricular fibrillation or at least increase the susceptibility of the heart to such a condition, while others consider that it prevents auricular fibrillation or causes established fibrillation to cease.

Knoll (1), Hewlett (2), Winterberg (3, 4, 5), and Cushny (6) point out that vagus stimulation increases the susceptibility of the heart to auricular fibrillation, and that faradization of the nerves alone may sometimes produce the condition in the mammalian heart. Winterberg (4, 5), who has investigated this question more thoroughly than anyone else, states that a weaker faradizing current is required to throw the auricles into fibrillation when combined with vagus stimu-

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lation or when drugs which raise the vagus tone have been administered. than when the auricles alone are stimulated. He also says that vagus stimulation may fix an auricular fibrillation set up by a short period of faradization, so that it continues after auricular faradization is removed.

McWilliams (7), Fischel (8), Philips (9), Hirschfelder (10), Garrey (11), Lewis (12), and Cushny (6) state that vagus stimulation, on the other hand, arrests auricular fibrillation. Winterberg (4) has noted that stimulation of the vagi may both make the heart more susceptible to auricular fibrillation and also arrest it when established. McWilliams, Lewis, Winterberg, Rothberger and Winterberg (13), and Hewlett describe the effects which vagus stimulation has on established auricular fibrillation, and all agree that it causes the fibrillary movements to become more rapid, finer, and less easily seen. Rothberger and Winterberg consider this effect to be the same as that which causes a great diminution or disappearance of the P wave in the electrocardiogram of the normal heart-beat. Finally, Kronecker and Spillata (14) conclude that vagus stimulation does not affect auricular fibrillation.

METHODS.

The material upon which the present communication is based consists of twenty-three experiments on dogs. Medium sized or rather large animals, weighing from 6,800 to 17,000 grams, were used. As the experiments were varied, the number bearing on the various points considered is not always the same, and so will be mentioned in each instance. Records were obtained by means of the string galvanometer (Edelmann), and in one experiment by suspension curves from auricles and ventricles as well. Direct observation of the heart also proved useful.

The usual method of experimentation was as follows: The animal was etherized by intratracheal insufflation (Meltzer-Auer method), and German silver electrodes over cotton soaked with salt solution were applied to the right fore leg and the left hind leg. The string of the galvanometer was always adjusted so that one millivolt caused a movement of ten millimeters on the record. After an electrocardiogram had been taken, the chest was opened by means of a lateral incision through the third or fourth intercostal space. The right auricle was exposed by a small incision of the pericardium, and to it were attached, about five millimeters apart, two small clips wrapped separately in rubber tissue. From these clips light wires ran to the secondary coil of a Harvard inductorium. Both vagi were then dissected out in the neck, but not, as a rule, cut. The

right auricle was usually stimulated for fifteen seconds by a faradic current. If the auricles did not continue to beat tumultuously after the faradization ceased, it was repeated three times at intervals of one minute. If still unsuccessful, the vagi were cut and stimulation of the auricle was repeated in the same manner. Several minutes were allowed to elapse between the two sets of stimulations. If rapid, tumultuous auricular activity became established during the first set of stimulations, electrocardiograms were made before and after cutting the vagi, so that the effect of cutting the nerves could be observed. After cutting the vagi, the peripheral end of each nerve was laid in a shield electrode, which was protected by rubber tissue and dry gauze. The effect of stimulation of both the right and left vagi was investigated when the auricles were affected by faradization, as well as when the heart was beating normally. When the altered activity following auricular faradization could not be established so that it continued independently, vagus stimulation was begun immediately before ending auricular faradization. As the altered auricular activity almost always peristed during vagus stimulation, the effects of stimulating the nerves upon it could be studied in this way.

The strength of the faradizing current did not bear a constant relation to the length of time the auricles remained affected after faradization was ended. A current just strong enough to keep the auricles constantly in a state of tumultuous activity seemed as effectual as stronger currents in causing this activity to continue after faradization had ceased. With one two-volt cell, it was found in several experiments that when the secondary coil of the inductorium was out eighty or ninety millimeters, the current was just strong enough to maintain auricular fibrillation. To be well within this limit but to avoid very strong currents, the auricle was usually stimulated with the secondary coil sixty millimeters out. The vagi were stimulated by a faradic current from an inductorium, the secondary coil of which was extended fifty millimeters. The strength of this current is the same as that used by Cohn (15) in his experiments on the vagus control of the normally beating heart, so that the results of his experiments and those of this series are comparable.

The importance of considering the effect of stimulating each vagus separately has become obvious since Cohn (15) has shown that there is a definite difference in function between the two nerves almost constantly demonstrable in dogs. This difference is as follows: when the right vagus in the dog is stimulated, there is, in a large proportion of cases, stoppage of the entire heart, while when the left is stimulated, the auricles beat at a rate not greatly reduced while partial heart-block or ventricular asystole usually occurs. The effect of stimulation of the two vagi must therefore be considered separately.

RESULTS.

1. *The Effect of Faradization on Auricular Activity.*—The auricles always beat in a rapid tumultuous manner during faradization. In some dogs this altered rhythm ceased synchronously with the end of faradization if no other measures were employed, while in others it continued and became established. This altered rhythm became established by faradization alone in eleven experiments, while in four others its establishment was accomplished by combining auricular faradization with vagus stimulation. It continued in these fifteen experiments from five minutes to over an hour after faradization, thus giving opportunities for studying its character and the effect of vagus stimulation upon it.

Direct observation of the exposed heart showed that the auricular activity following faradization consisted in very rapid movements, apparently contractions of the whole auricles, which were sufficient to produce definite movements of the recording tambour attached to the auricular myocardiograph (figure 11). Besides this rapid auricular tachycardia, fine fibrillatory movements in the various fibers could be seen. During the right vagus stimulation, the effect of which will be discussed later, the coarser movements ceased and the typical fine fibrillations persisted, and when the stimulation was removed the coarser movements could be seen definitely gradually returning and being coexistent with the fine fibrillations. This direct observation was confirmed by my colleague, Dr. Cohn, who first observed this phenomenon in one of my experiments.

The electrocardiogram (figure 7) shows no waves which resemble those caused by the normal auricular contractions, but between the

rapidly and irregularly recurring ventricular complexes, irregular waves of various sizes occur usually at a rate averaging 500 to 600 per minute. The waves may be four to five millimeters in height, but the larger waves are often interrupted by much smaller, finer, and more rapid movements of the string. The irregularities in the size and shape of the ventricular complexes are probably caused by various degrees of coincidence with the waves of auricular activity.

It is evident that the altered activity of the auricles usually resulting from faradization is not true fibrillation alone, but consists of two different forms of cardiac activity occurring simultaneously in the auricles; namely, tachycardia and fibrillation.

The auricular activity following faradization has been described and recorded by electrocardiograms by Rothberger and Winterberg and by Lewis. The records obtained by these observers agree closely with ours. From their direct observations they describe different grades of fibrillation, varying in extent of auricular movements. Lewis states that actual correspondence between the auricular activity and the electrocardiograms may be found only rarely, while Rothberger and Winterberg consider that no correlation can be made out. Beside these various grades of fibrillation Rothberger and Winterberg describe finally what they call *unreines Schlagen.* This form of auricular activity consisted of definite contractions of the auricles which affected at least the main mass of the musculature, together with either fibrillatory movements seen more or less distinctly, especially on the margins of the auricles, or weak peristaltic waves running across the auricles, occurring at the same time. This peculiar combination of the normal beat and delirium caused no corresponding impression on the electrocardiogram. The auricular wave was usually somewhat split, and here and there the rhythm was disturbed. They do not state how frequently this type of auricular activity was seen or under what conditions it occurred, and no further comment is made upon it. It is evident from their description that Rothberger and Winterberg have observed the same form of auricular activity which resulted almost constantly from auricular faradization in our experiments. The various grades of fibrillation which these observers and Lewis describe did not appear in our experiments except under conditions to be discussed,

although a pure auricular tachycardia, as observed by Hirschfelder and others, followed faradization sometimes in some of our experiments. The rapid auricular activity may be conveniently designated tachycardia in this paper, as this was practically always the most obvious effect of auricular faradization.

2. *The Influence of Cutting Both Vagi.* (*a*) *On the Susceptibility of the Normally Beating Heart to Auricular Faradization* (*Fourteen Experiments*).—Prolonged auricular tachycardia was more readily produced by faradization after cutting both vagi in three experiments, while in ten no change in the susceptibility of the auricles to faradization could be detected. In one tachycardia seemed more readily produced before than after cutting the nerves. The protocol of experiment 12 shows the positive influence that cutting the vagi may have.

11:20 A. M.	Auricular faradization, 15 seconds.	Tachycardia for 10 seconds after end of faradization.
11:21 A. M.	Auricular faradization, 15 seconds.	Tachycardia for 3 seconds after end of faradization.
11:22 A. M.	Auricular faradization, 15 seconds.	Tachycardia for 8 seconds after end of faradization.
11:23 A. M.	Right vagus tied and cut.	
11:24 A. M.	Left vagus tied and cut.	
11:27 A. M.	Auricular faradization, 15 seconds.	Tachycardia for 19 minutes after end of faradization. Normal beat returned only after four vagus stimulations.

(*b*) *On the Heart with Established Auricular Tachycardia* (*Eight Experiments*).—In these experiments prolonged tachycardia was established with the vagi intact, so that records could be obtained before and after they were cut. This procedure had apparently very little effect on the auricular activity. In six experiments no effect could be detected in the electrocardiograms, while in one the waves of auricular activity were considerably slowed and in another rendered larger and more distinct. The ventricular rate was increased in four experiments, while in three there was practically no change. In the one other experiment, no conclusions can be drawn, as other procedures may have influenced the ventricular rate. In several experiments there was seen a gradual increase in ventricular rate

as auricular tachycardia continued, so caution is necessary in interpreting these results, but in three, at least, of the four positive experiments, the results seem quite definite. This is seen especially well in experiment 13 where the ventricular rates during auricular tachycardia were as follows:

86 per minute 1 minute after onset.
160 per minute 5 minutes after onset.
168 per minute 7 minutes after onset (figure 1).
Both vagi cut 9 minutes after onset.
300 per minute 12 minutes after onset (figure 2).
240 per minute 20 minutes after onset.

When the heart was beating normally, cutting the vagi caused a definite increase in the rate of the heart-beat in thirteen of seventeen experiments.

3. *The Influence of Stimulation of Each Vagus Nerve.* (*a*) *On the Auricular Activity.*—Stimulation of the right vagus nerve (nineteen experiments) had a definite and almost constant effect on the auricular tachycardia, which could be observed by watching the heart directly and by changes in the electrocardiograms. Suspension curves were made in one experiment in which the heart behaved in a manner entirely typical of the other experiments. The auricular activity could be seen to undergo the marked change mentioned above. Instead of the auricles moving about rapidly as a whole, they became practically stationary, allowing the true fibrillatory contractions of the various muscles fibers to be plainly seen. They were similar to those observed in the ventricles when in fibrillation. In the electrocardiograms the undulations representing auricular activity became more rapid, blurred, and often almost disappeared. The large waves, sometimes four to five millimeters in height, rarely appeared (figures 5, 8, 12). In the experiment in which suspension curves were also made of the right auricle and right ventricle, it was found that the auricular tachycardia produced movements of the recording tambour nearly as distinct as those produced by the normally beating heart (figures 10 and 11). When the right vagus was stimulated (figure 12), the waves of the auricular suspension curve became smaller and smaller and finally disappeared, synchronously with the replacement in the electrocardiogram of very fine

small waves of true fibrillation for the well defined coarse waves of auricular tachycardia. Finally, it may be mentioned again that soon after right vagus stimulation, distinct contractions of the entire auricles could be seen accompanying but not replacing the fine fibrillatory movements in the auricular walls. It is evident, therefore, that when the right vagus was stimulated, it exerted its inhibitory influence on the auricular tachycardia, causing it to cease, while the true fibrillatory movements were apparently unaffected. This effect of right vagus stimulation occurred in seventeen of the nineteen experiments (89.5 per cent.), and may therefore be considered characteristic. It is apparently similar to that which has been described by other experimenters already mentioned. In one of the two instances where the typical effect was not observed, the character of the record changed in an unusual manner. At first the waves were blurred and very irregular. Then separate, distinct little waves, resembling the normal P waves, appeared at a rate of 560 per minute, and continued to occur as long as the vagus was stimulated. Here the auricular tachycardia apparently succeeded in reasserting itself. In the second instance no change could be observed in the auricular activity. In this experiment even the normally beating heart did not react to right vagus stimulation in the usual manner, as a distinct delay occurred between the time of onset of stimulation and stoppage of the heart.

The effect of left vagus stimulation on auricular fibrillation was studied in seventeen of the nineteen experiments in which right vagus effects were observed. A constant difference was observed between the influence of right and of left vagus stimulation in twelve of these seventeen experiments (70.6 per cent.). During left vagus stimulation direct inspection showed that the coarser movements of the auricles were not disturbed, but appeared sometimes perhaps even more distinctly. It was difficult to determine with certainty whether the fibrillatory movements ceased or were influenced. In some of the electrocardiograms there was seen slight blurring and quickening in rate of the waves of auricular activity similar to, but always less than, that seen with right vagus stimulation in the same animal. In other records the waves of auricular activity occurred more regularly, uniformly, and distinctly during

left vagus stimulation than before, and only the larger waves appeared, the small, fine rapid movements of the string which interrupted the larger waves practically disappearing (figures 6, 9, and 13). While some records, those first mentioned, indicate that the difference between the effects of right and left vagus stimulation seems to be one of degree rather than of kind, the others show that the type of response to the two nerves seems different. The distinctness and regularity with which the waves of auricular activity appeared may have been due to the removal of the confusing ventricular complexes, but the records suggest strongly that left vagus stimulation affected the auricular activity in an opposite manner from right vagus stimulation, and a change was produced which, instead of blurring and quickening the auricular waves, rendered them clearer, more uniform, and more regular.

The suspension curve from the auricles (figure 13) shows that the coarser auricular activity was not disturbed during left vagus stimulation, while during right vagus stimulation the suspension curve gave no evidence of motion. It seems evident, therefore, that stimulation of the left vagus often had practically no influence on the auricular tachycardia, while there is some evidence that it exerted an inhibitory influence on the true fibrillation.

In four experiments no definite difference could be made out between the effect of the two vagi on the auricular activity resulting from faradization. In three of these fairly marked changes occurred when each nerve was stimulated, while in the fourth the effect of each nerve was very slight.

The effect of stimulation of each nerve on the normally beating heart was studied in seventeen experiments, and in twelve of these (70.6 per cent.) the characteristic differences between the effects of stimulation of the two nerves were seen, just as described by Cohn (figures 3 and 4). Although the difference in effect of stimulation of the two nerves on the normally beating heart is more striking, it does not appear to occur more constantly than the difference in effect on auricular activity after faradization.

The difference in the effects of stimulation of the two vagi are seen in the figures. The curves in figures 3 to 6 are from one experiment (No. 18) and show the action of the two vagi on the heart,

both when it was beating normally and when auricular tachycardia was present. In this experiment prolonged auricular tachycardia could not be established, so vagus stimulation was begun before the end of auricular faradization. The curve in figure 7 (experiment 11) is an electrocardiogram obtained after the auricles had been faradized, and figures 8 and 9 from the same experiment show the changes in the electrocardiograms which resulted from vagus stimulations. Figures 10 to 13 show suspension curves from the auricles and ventricles, and illustrate the changes in auricular activity which have been described.

The results of the various experiments indicate that the length of time that tachycardia had been established did not apparently influence the vagus action upon it, and that the difference in effect of stimulation of the two nerves was observed regardless of which nerve was stimulated first.

(*b*) *On Reëstablishment of the Normal Sequential Beat* (*Thirteen Experiments*).—It was frequently seen that after auricular tachycardia had become established and had continued for several minutes without showing any signs of ceasing spontaneously, it would cease several seconds after the end of stimulation of one of the vagi (figure 14). It was concluded from the constant behavior of the dog's heart in this respect that vagus stimulation was the cause of the reëstablishment of the normal sequential beat. Of thirteen experiments, stimulation of each nerve was followed by the reëstablishment of the normal beat in seven. Left vagus stimulation alone produced this result in three, right vagus stimulation produced it in one, while in two experiments the normal beat never returned as a result of vagus stimulation. In other words, right vagus stimulation caused cessation of the abnormal auricular activity in 61.5 per cent. of the experiments, while left vagus stimulation had this effect in 76.9 per cent. Although numerically considered, this difference in the two nerves is not great, it is clear that stimulation of the left vagus was more effectual in reëstablishing the normal sequential beat than that of the right. Vagus stimulation did not usually seem to produce any permanent change in the susceptibility of the auricles to faradization, although in two or three experiments the auricles apparently became more resistant to faradization after the vagi had been stimulated a number of times.

(*c*) *On the Susceptibility of the Auricles to Faradization.*—In the experiments in which prolonged auricular tachycardia could not be established by auricular faradization alone, the influence of vagus stimulation in aiding the auricles to maintain prolonged tachycardia could be observed. The method of determining this influence was to throw the auricles into tachycardia by faradization for fifteen seconds, and to begin vagus stimulation just before ending auricular faradization. Vagus stimulation was continued for five to eight seconds. Twice in ten experiments right vagus stimulation caused the tachycardia to become established and to continue for some minutes after the vagus stimulation ceased. The same result was obtained in two other experiments with left vagus stimulation. In seven of the eight experiments in which faradization combined with right vagus stimulation did not succeed in establishing the tachycardia, the auricles always remained in a state of tachycardia as long as the nerve was stimulated. It seemed quite evident that the auricular tachycardia was maintained by vagus action. In the remaining experiment tachycardia continued during three out of four vagus stimulations, so it can be said that when the auricles were thrown into a state of tachycardia by auricular faradization, they continued almost invariably in such a state as long as the right vagus was stimulated. With left vagus stimulation this result was obtained in but five of eight experiments, and in three experiments in which right vagus stimulation maintained auricular tachycardia, left vagus stimulation, although active in other respects, failed to do so. It appears that stimulation of the right vagus nerve was more effectual than stimulation of the left in aiding the auricles to maintain the activity set up by faradization, a fact that was especially noted in the protocols of several experiments.

(*d*) *On Initiating Auricular Tachycardia.*—In two experiments on hearts in which auricular tachycardia was readily established by faradization, it was also initiated by right vagus stimulation alone. In one (No. 25) it began on two occasions during vagus stimulation (figure 15), and in the other (No. 13) this occurred two seconds after the stimulation ceased (figure 16). This first experiment (No. 25) is especially interesting because auricular tachycardia was stopped as well as started by right vagus stimulation. Twenty-

four minutes after the tachycardia had been established by right vagus stimulation alone (figure 15), it ceased four seconds after stimulation of the same nerve (figure 14). This result was obtained twice in this experiment with right vagus stimulation, but stimulation of the left nerve was also followed by a return of the normal beat two minutes after auricular tachycardia had been set up by right vagus stimulation alone.

Stimulation of the left nerve alone never caused auricular tachycardia in these experiments, although it did follow left vagus stimulation in one case in a long series of vagus stimulations by Dr. Cohn.

The influence of vagus stimulation on the ventricles during the type of cardiac activity which results from auricular faradization will not be considered in this paper.

DISCUSSION.

The auricular activity which follows faradization of the auricles is apparently the result of a change in the physiological properties of the auricular musculature. This change seems to alter the excitability and the power of stimulus formation in the auricles and finds expression in the establishment synchronously of auricular tachycardia and true fibrillation. This altered auricular activity persists after faradization of the auricles has been withdrawn, indicating that the altered cardiac properties do not immediately return to their normal state as soon as the exciting cause is removed.

The normal tonic vagus activity has in some animals a definite control over these cardiac properties under the conditions of our experiments, as cutting the vagi sometimes increases the susceptibility of the auricles to faradization. After the auricles take on the activity resulting from faradization, the tonic vagus action on the auricles seems very slight, as but little if any effect can be detected when the vagi are cut during auricular tachycardia. The influence of this procedure on the ventricular activity is, however, in some experiments quite definite, and results in a marked increase in ventricular rate. The result depends obviously upon a change in conductivity in the auriculoventricular conducting system. The depression of auriculoventricular conduction is one of the most obvious

activities of the vagi, and when this depression is removed, the ventricles are stimulated to contraction by a larger number of impulses from the rapidly contracting auricles than when the vagi are intact. That this increase in ventricular rate is not constant when the vagi are cut indicates that conductivity is not always depressed by tonic vagus action.

The effects of vagus stimulation on the auricular activity resulting from faradization are in accord with the effects which stimulation of the two nerves has on the normally beating heart. Stimulation of the right vagus stops the auricular tachycardia just as it stops the auricles in the normally beating heart, and stimulation of the left nerve fails to do so in both instances. True auricular fibrillation obviously is not controlled by right vagus stimulation, while there is insufficient evidence to allow any definite statement as to the effect of left vagus stimulation upon it. The electrocardiograms seem to show, however, that auricular fibrillation may be inhibited by left vagus stimulation while the tachycardia proceeds undisturbed.

Auricular tachycardia in man may sometimes be controlled by pressure over the right vagus nerve, as will be shown by electrocardiograms to be published shortly by my colleagues, Drs. Cohn and Fraser, while Draper and I (16) have published electrocardiograms which indicate that right vagus pressure does not influence auricular fibrillation in man.

The mechanism by which the normal sequential beat is restored a few seconds after vagus stimulation is difficult to understand. Left vagus stimulation seems somewhat more effectual in producing this result than right vagus stimulation, so that it cannot come from inhibitory influences of the auricular tachycardia. More facts are needed before an explanation of this phenomenon can be attempted.

Vagus stimulation increases the susceptibility of the auricles to faradization. This is indicated by the fact that in animals whose auricles are resistant to faradization, the abnormal auricular activity continues after faradization as long as one of the vagi is stimulated. This effect probably results from the inhibitory influence of vagus stimulation on the normal auricular activity. As long as the normal pace-maker is depressed, there is probably a diminished tendency for the abnormal auricular activity to be superseded by the normal

sequential beat. The more marked influence which the right vagus has on the normal auricular activity explains why it is more effectual in holding the auricles in the abnormal activity than the left nerve. It may be that it is through this same mechanism that the abnormal auricular activity can be originated by vagus stimulation alone. If the auricles are in a state very favorable to the establishment of tachycardia and fibrillation, inhibition of the normal pace-maker alone may suffice for the establishment of the abnormal auricular activity. The fact, however, that the tachycardia may come on during right vagus stimulation without showing the usual effect of the stimulation (figure 15), or that it may come on at a time after the stimulation when the normal pace-maker usually reasserts itself (figure 16), does not lend support to this possibility. It is not clear also why a short vagus stimulation following the faradization should assist in the establishment of the abnormal activity of the faradized auricle as an independent auricular activity.

SUMMARY AND CONCLUSIONS.

An abnormal auricular activity is produced by faradization of the right auricle of the dog, which frequently becomes established and continues for varying periods of time after faradization is discontinued. This auricular activity consists of a rapid auricular tachycardia coexisting with true auricular fibrillation. In some dogs the auricles are thrown into this abnormal activity more readily by faradization after the vagi have been cut than before. Cutting the nerves has little or no effect on the abnormal auricular activity, but the ventricular rate may be much increased if the vagi are cut after the abnormal auricular activity has been established, apparently because of an improvement in the auriculoventricular conductivity.

Stimulation of the right vagus nerve changes the character of the activity of the faradized auricles by inhibiting the auricular tachycardia while the fibrillation is uninfluenced. Stimulation of the left vagus nerve has little or no apparent inhibitory effect on the auricular tachycardia, but has possibly an inhibitory effect on the auricular fibrillation.

Vagus stimulation increases the susceptibility of the auricles to faradization. The abnormal activity set up by faradization may be

EXPLANATION OF PLATES.[1]

Plate 71.

Fig. 1. Experiment 13. Auricular tachycardia, vagi intact. Ventricular rate, 168 per minute.

Fig. 2. Experiment 13. Auricular tachycardia, vagi cut. Ventricular rate, 300 per minute.

Fig. 3. Experiment 18. Normal beat. Effect of right vagus stimulation of 3.2 seconds' duration.

[1] Time marking in all curves is in 1/5 and in 1/50 of a second.

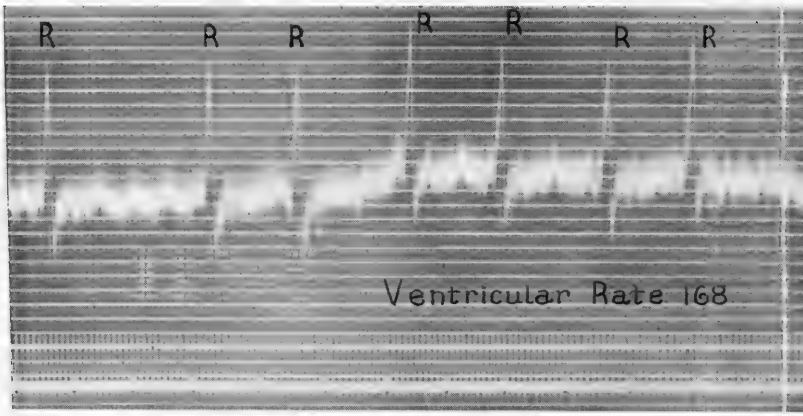
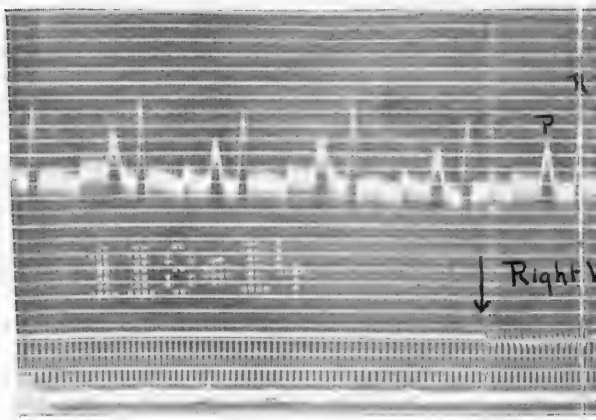


FIG.



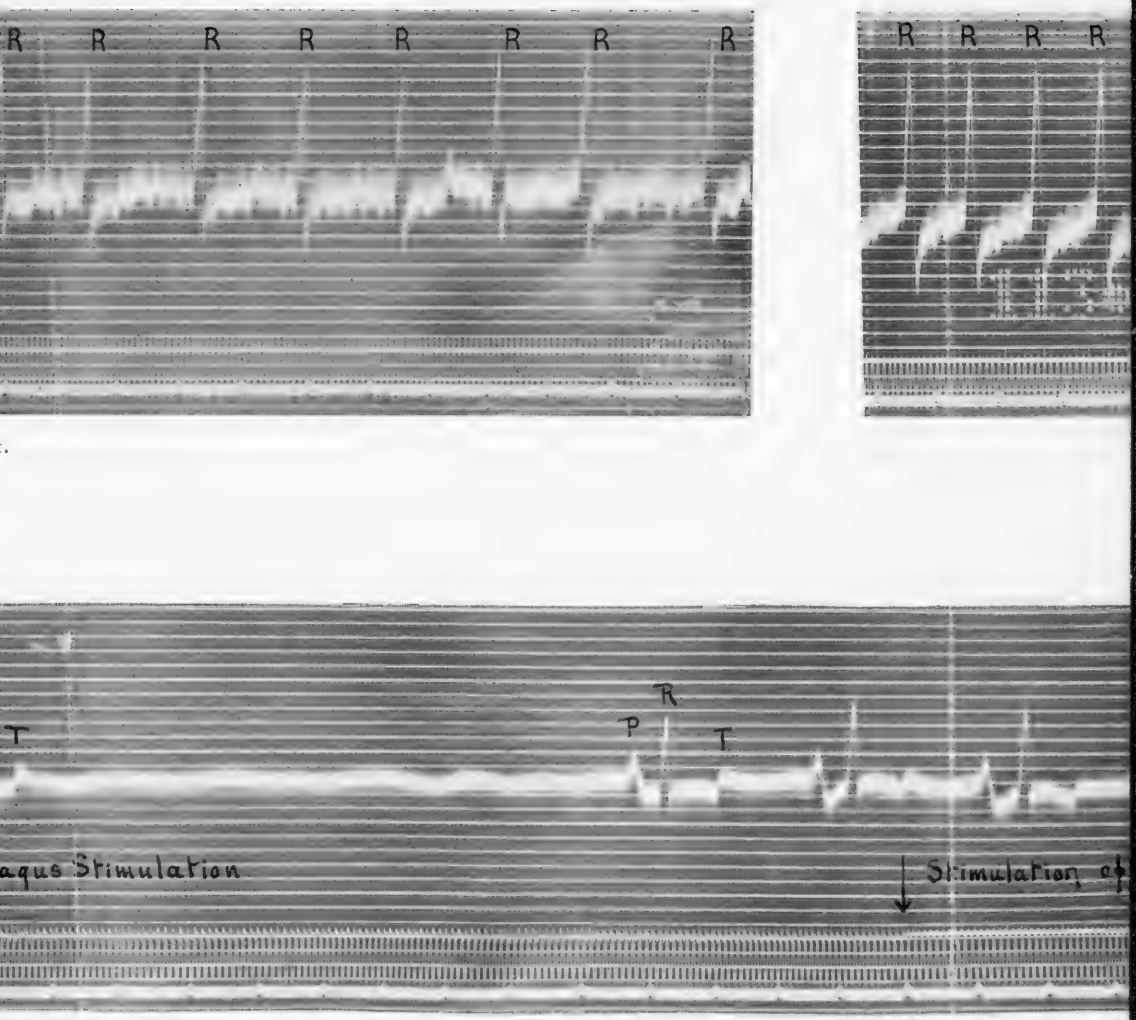


FIG. 3.

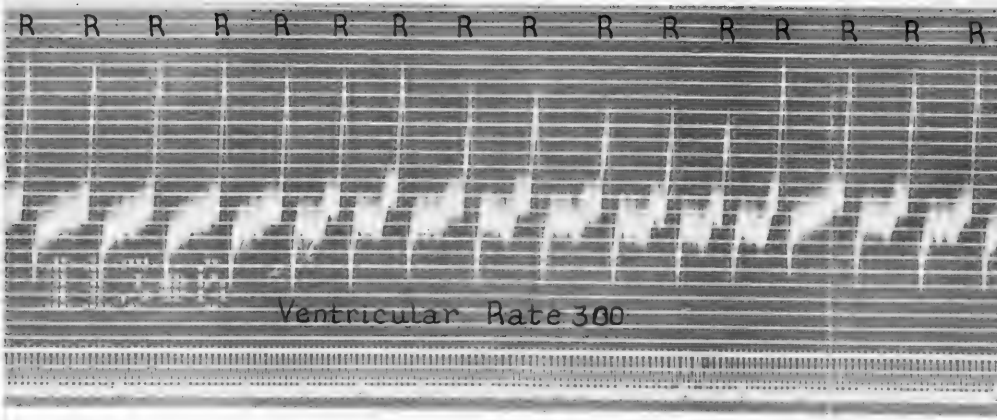


FIG. 2.

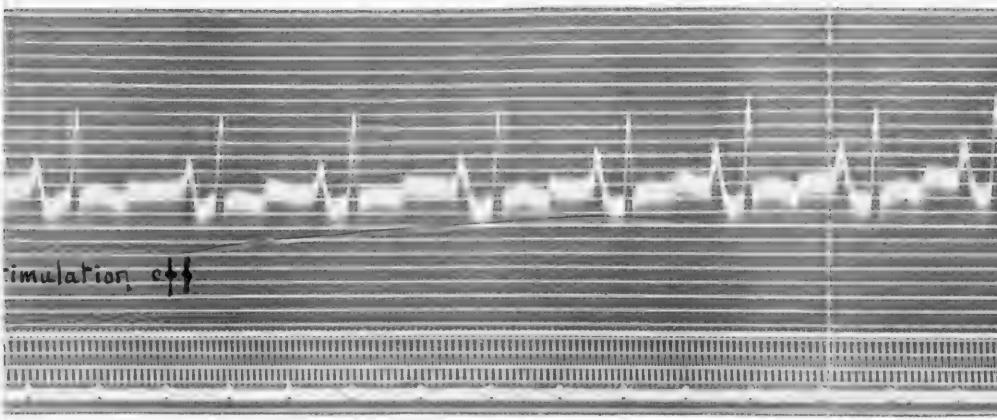
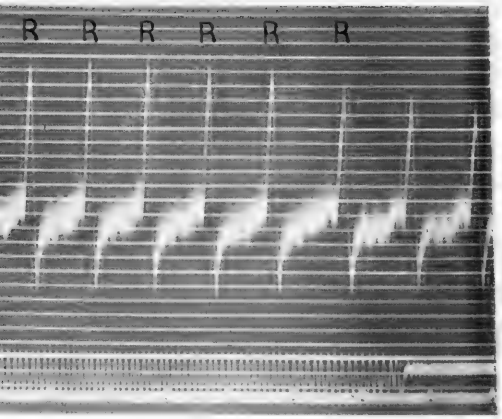
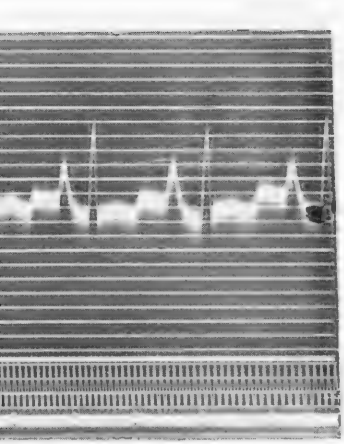


PLATE 71.



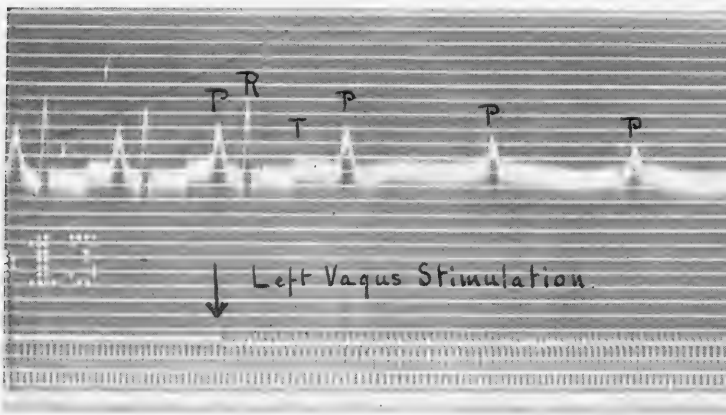


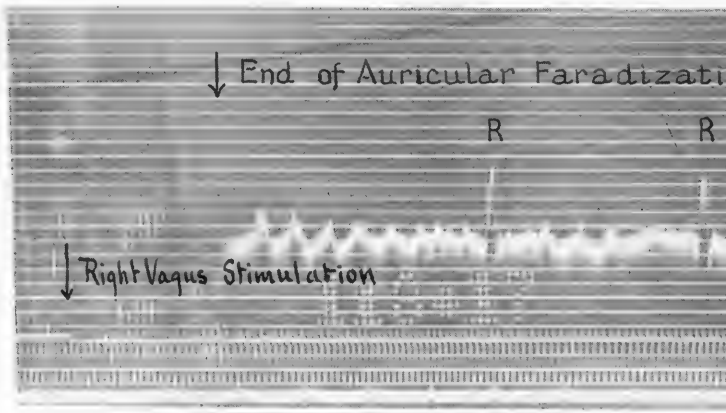
(Robinson : Influence of Vagus Nerves.)

PLATE 72.

FIG. 4. Experiment 18. Normal beat. Effect of left vagus stimulation of 6.6 seconds' duration.

FIG. 5. Experiment 18. Auricular tachycardia. Effect of right vagus stimulation of 7.6 seconds' duration, begun during auricular faradization.


P
R
T
P
P
P
Left Vagus Stimulation


End of Auricular Faradizati
R
R
Right Vagus Stimulation

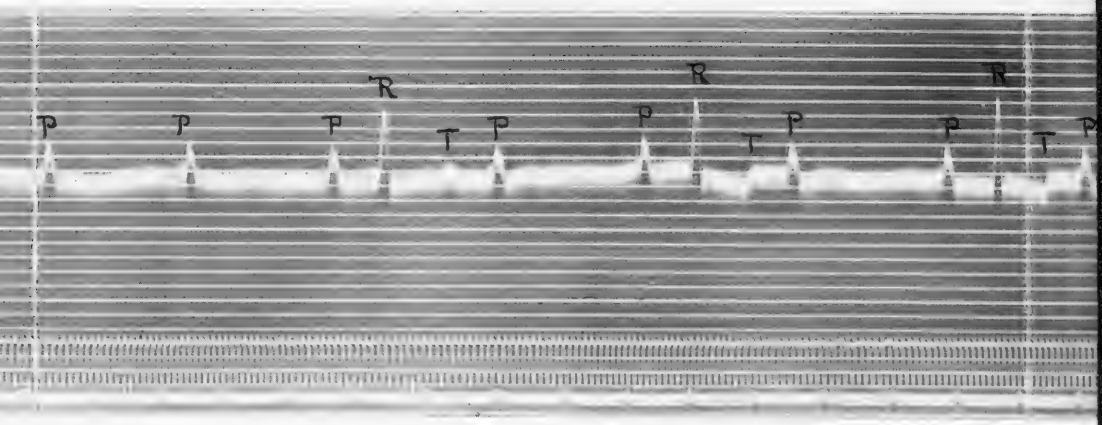


Fig. 4.

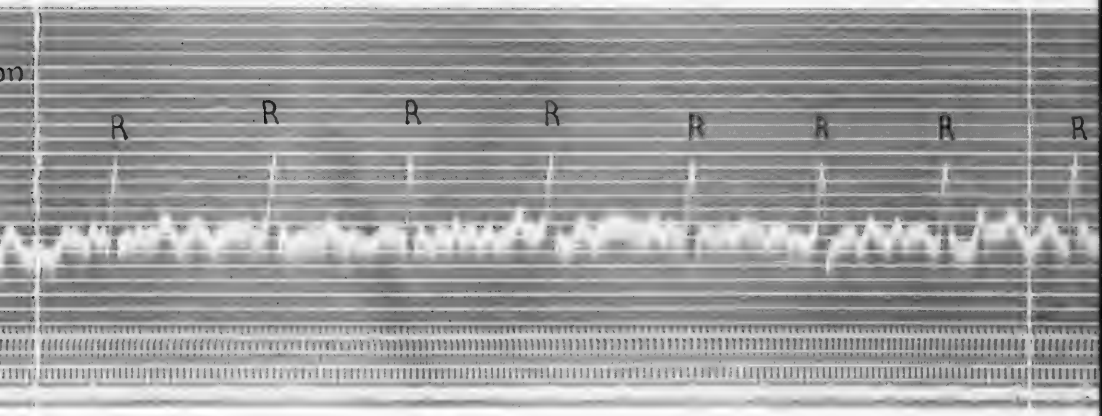
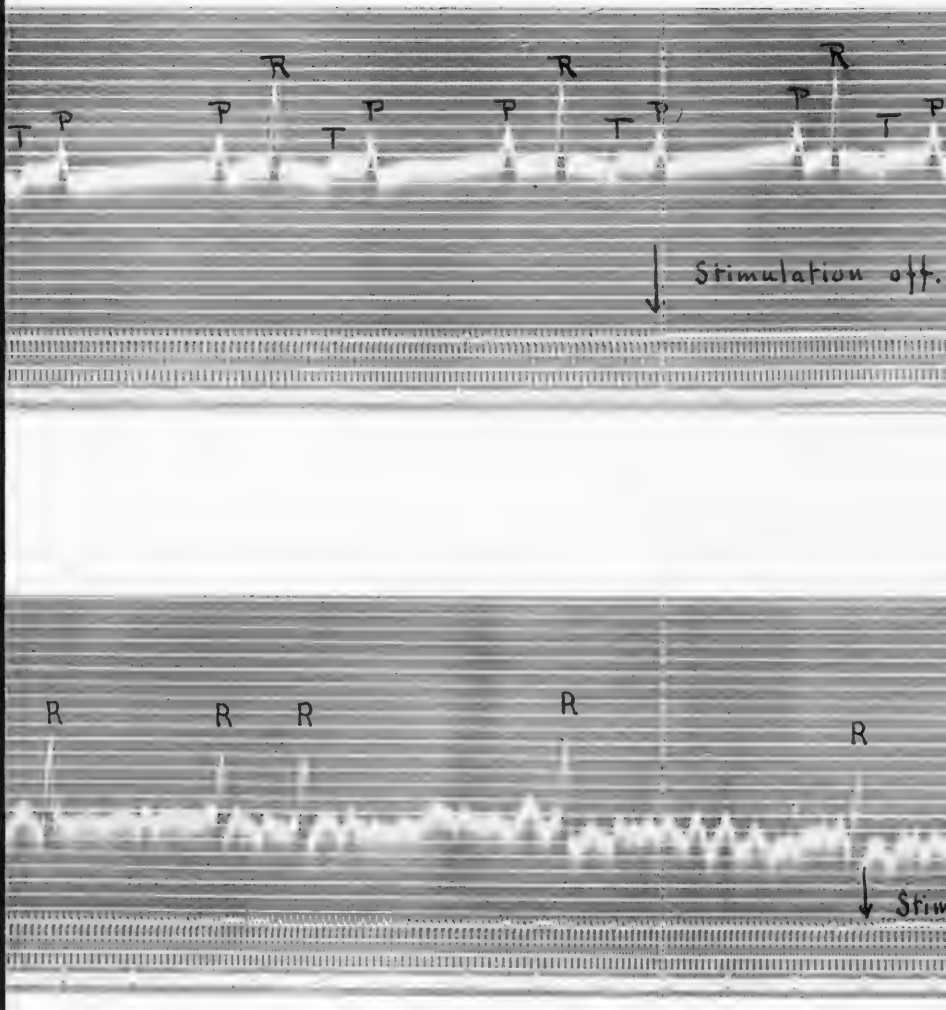


Fig. 5.



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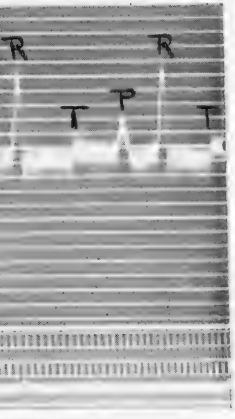
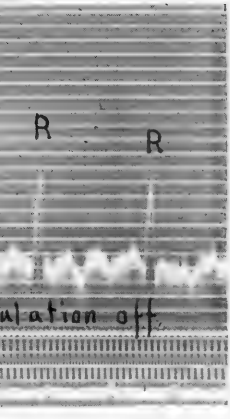


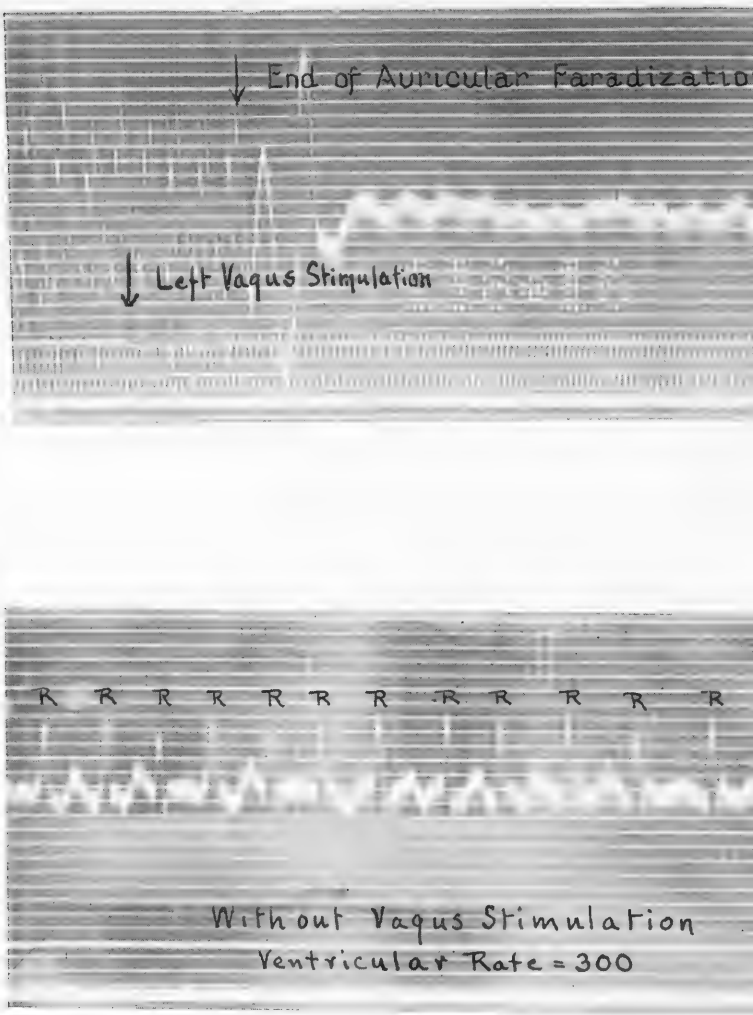


: Influence of Vagus Nerves.)

PLATE 73.

FIG. 6. Experiment 18. Auricular tachycardia. Effect of left vagus stimulation of 5.4 seconds' duration, begun during auricular faradization.

FIG. 7. Experiment 11. Auricular tachycardia without vagus stimulation.


End of Auricular Faradizatio
Left Vagus Stimulation
R R R R R R R R R R R R
Without Vagus Stimulation
Ventricular Rate = 300

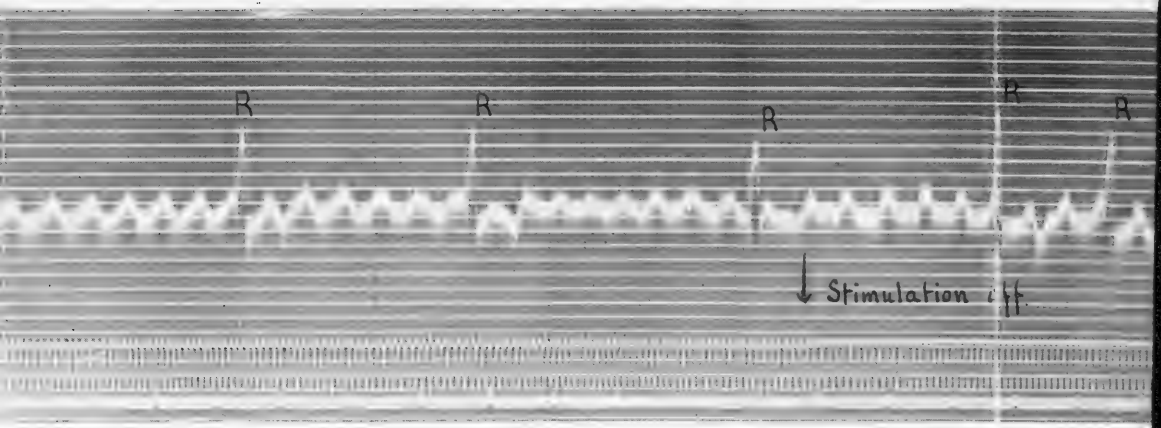


FIG. 6.

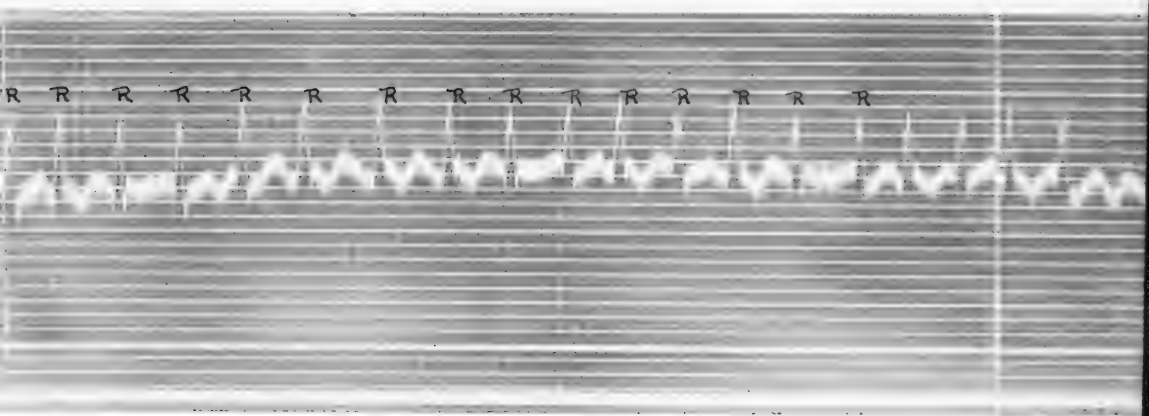
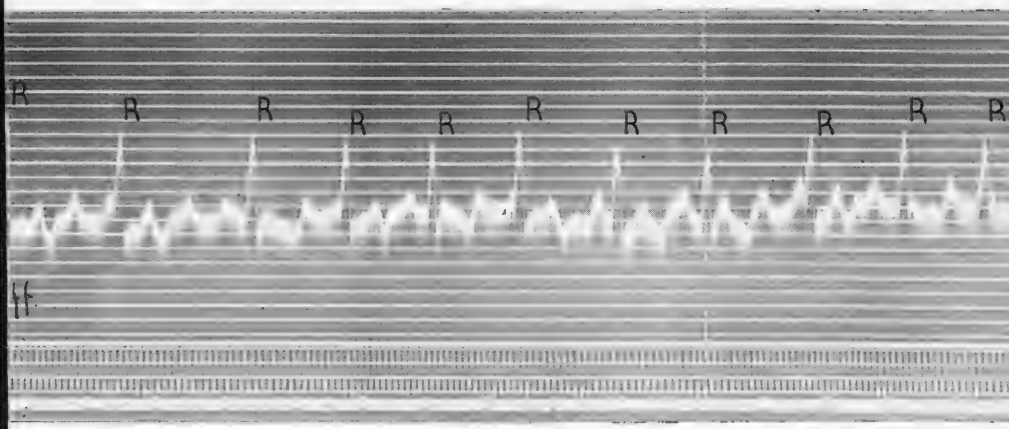


FIG. 7.



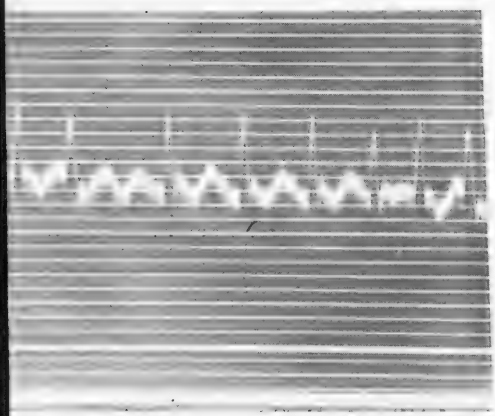


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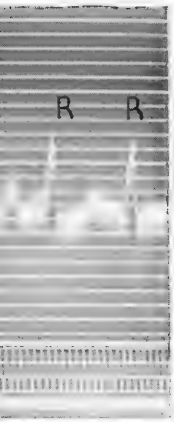
PLATE 73.

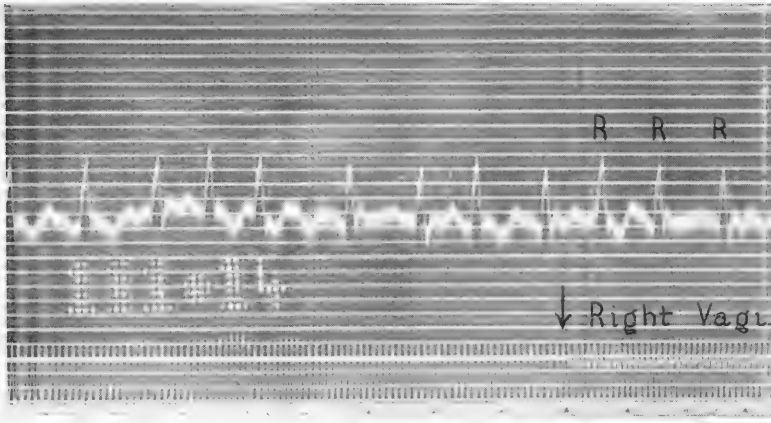


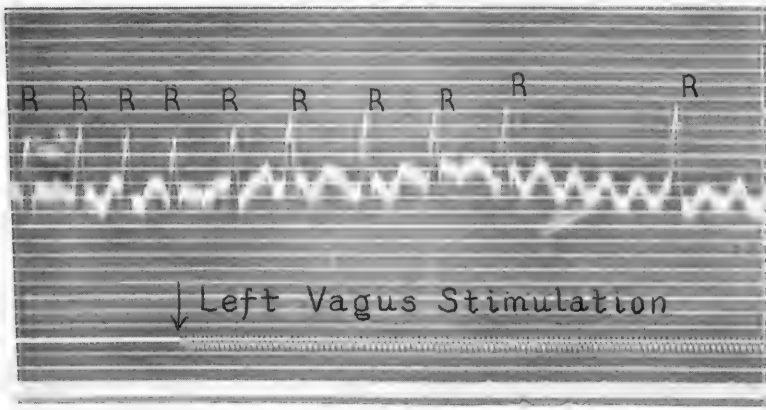
uence of Vagus Nerves.)

PLATE 74.

FIG. 8. Experiment 11. Auricular tachycardia. Effect of right vagus stimulation of 5.6 seconds' duration, begun ½ minute after the onset of auricular tachycardia.

FIG. 9. Experiment 11. Auricular tachycardia. Effect of left vagus stimulation of 6.2 seconds' duration, begun 2 minutes after the onset of auricular tachycardia.


R R R
↓ Right Vagu


R R R R R R R R R R
↓ Left Vagus Stimulation

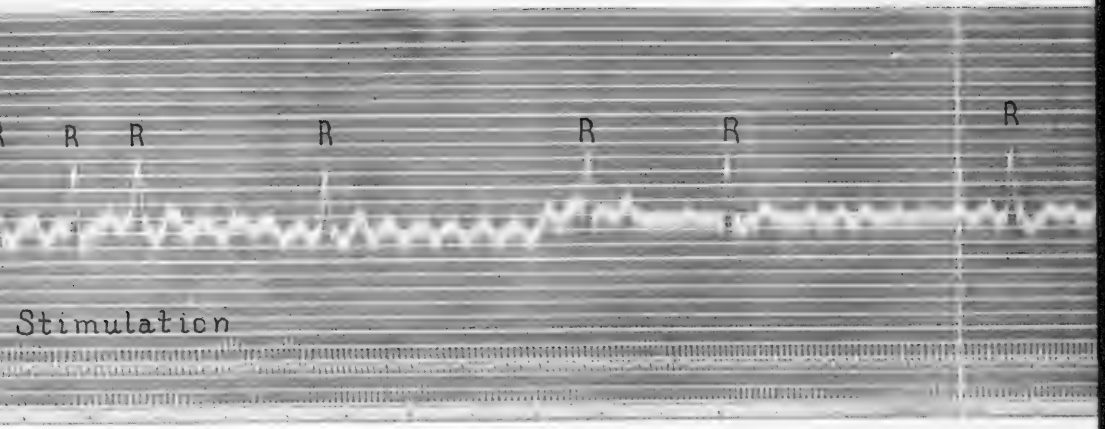


FIG. 8.

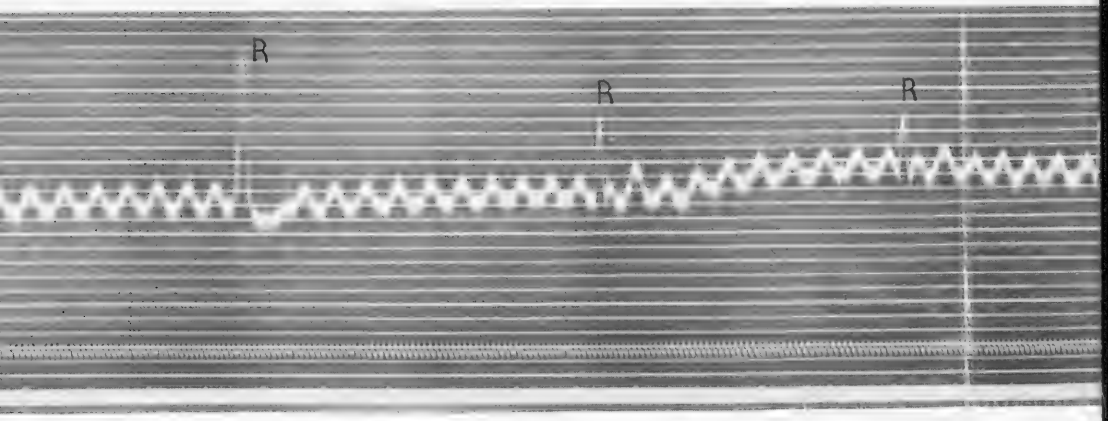
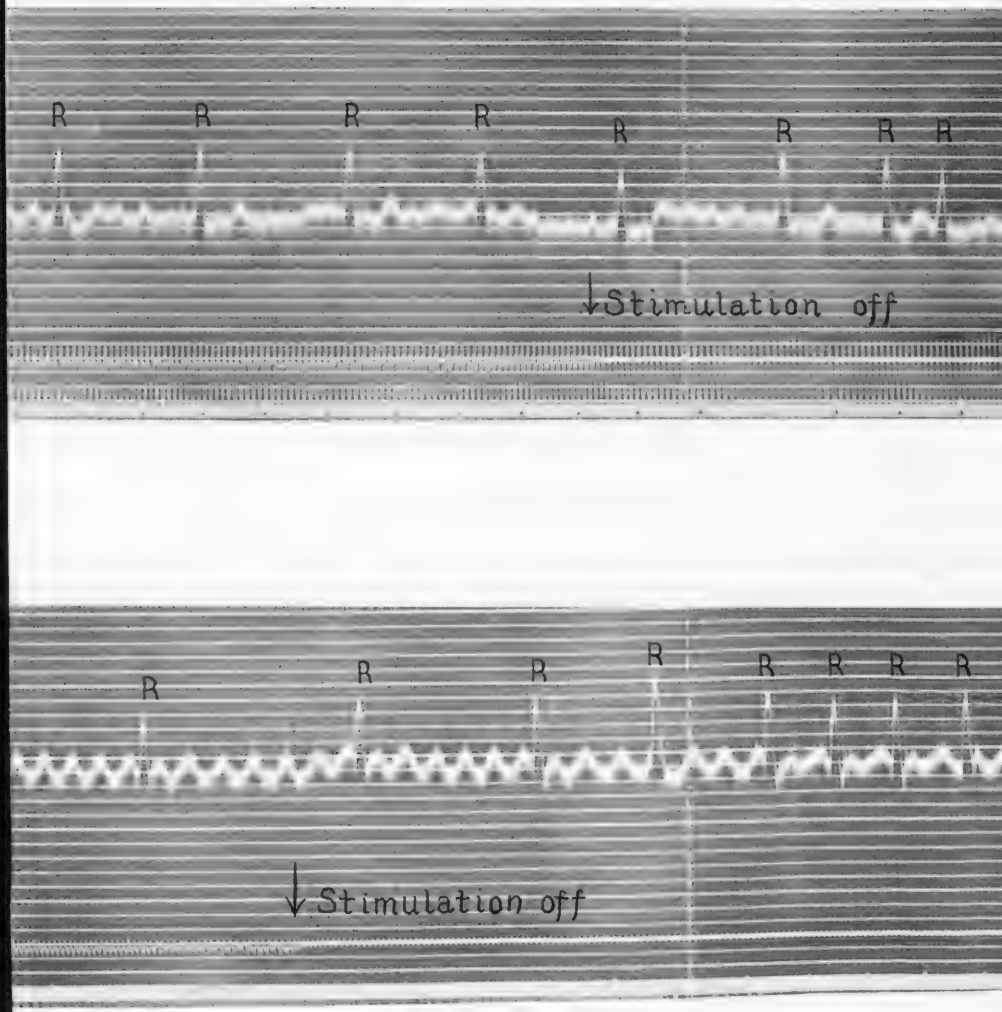


FIG. 9.



(Robinson : I

PLATE 74.

nfluence of Vagus Nerves.)

PLATE 75.

FIG. 10. Experiment 27. Normal beat. Suspension curves from auricles and ventricles and electrocardiogram.

FIG. 11. Experiment 27. Auricular tachycardia without vagus stimulation. Auricular rate, 550, as shown by the suspension curve and by the electrocardiogram.

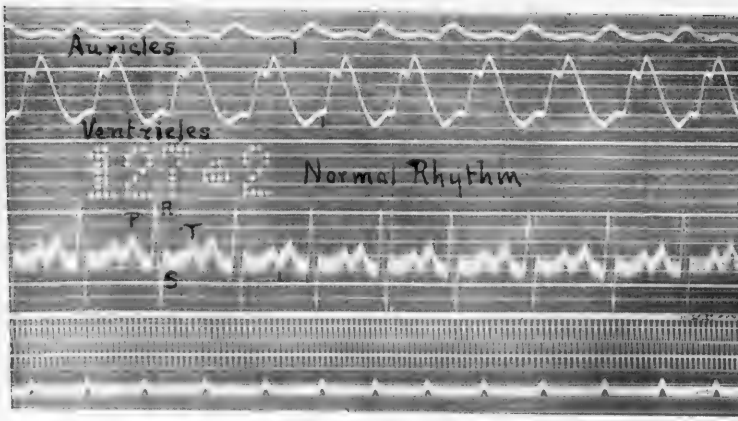

Auricles
Ventricles
Normal Rhythm
P
R
T
S

FIG. 10.

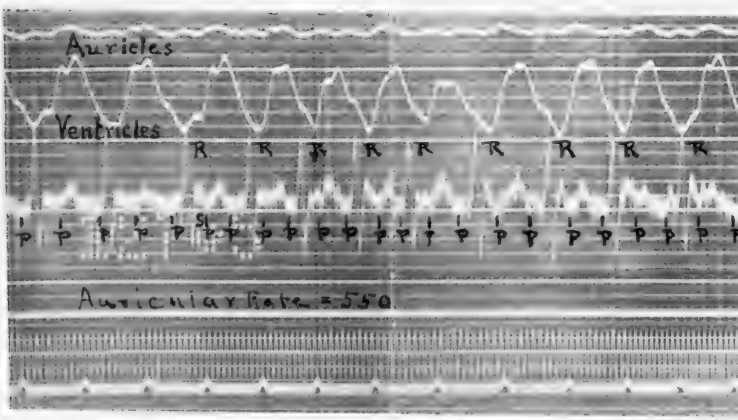

Auricles
Ventricles
Auricular Rate = 550

FIG. 11.

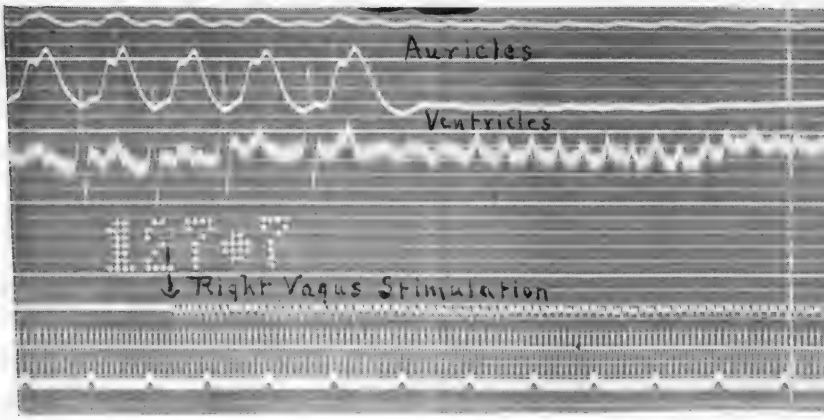
TE 75.

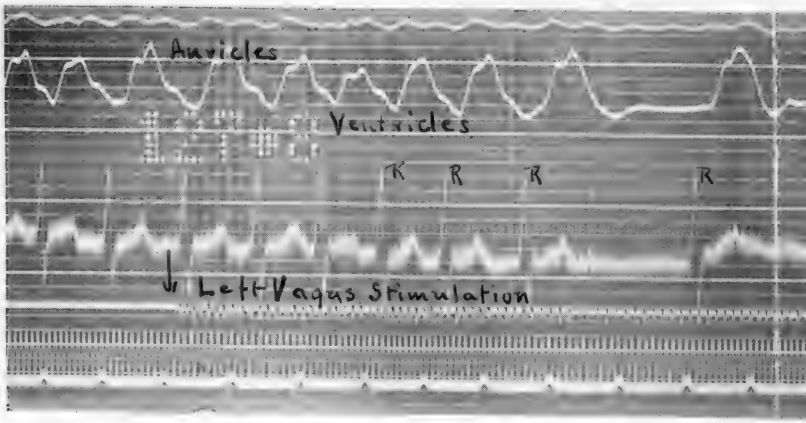
s Nerves.)

PLATE 76.

FIG. 12. Experiment 27. Auricular tachycardia. Effect of right vagus stimulation as shown by the suspension curve and electrocardiogram. True fibrillation during the period of ventricular asystole.

FIG. 13. Experiment 27. Auricular tachycardia. Effect of left vagus stimulation. Auricular rate before stimulation, 525. During stimulation, 523 per minute, as seen in suspension curve. The electrocardiogram is confused by an escape of the stimulating current.


Auricles
Ventricles
Right Vagus Stimulation


Auricles
Ventricles
R
R
R
R
Left Vagus Stimulation

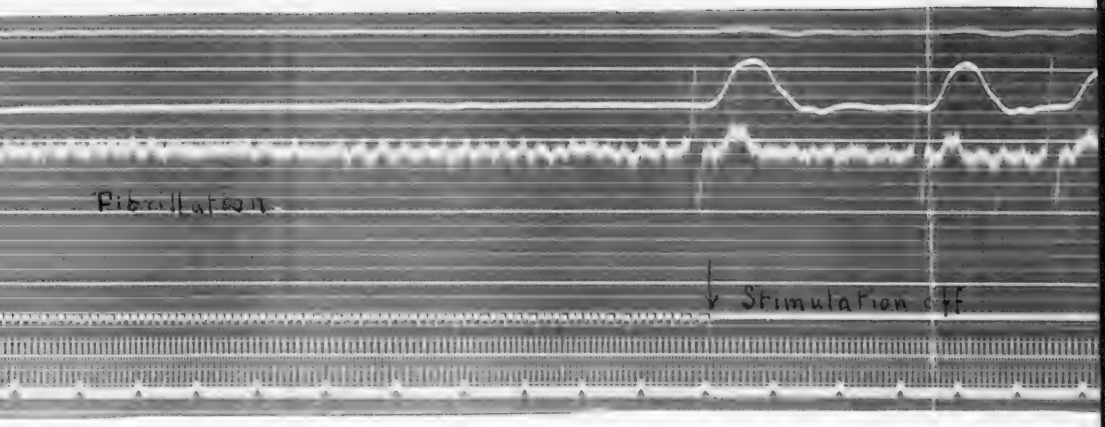


FIG. 12.

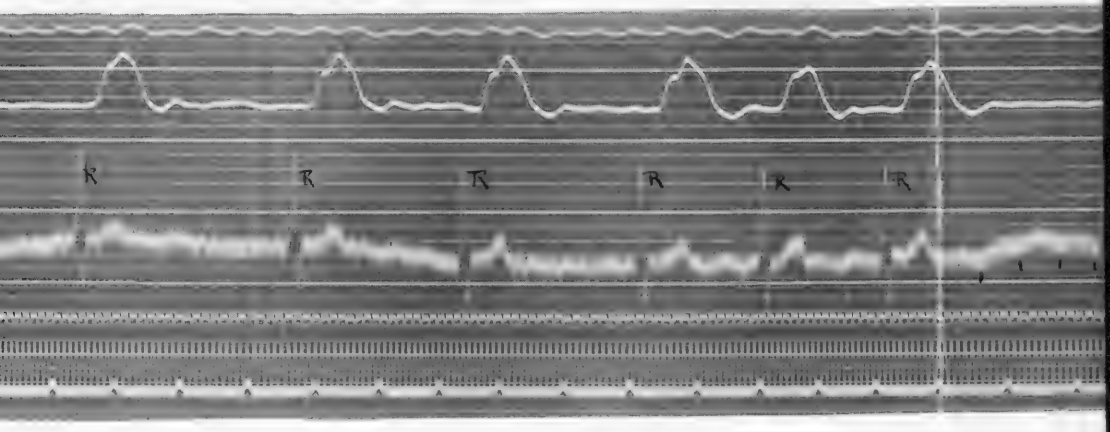


FIG. 13.

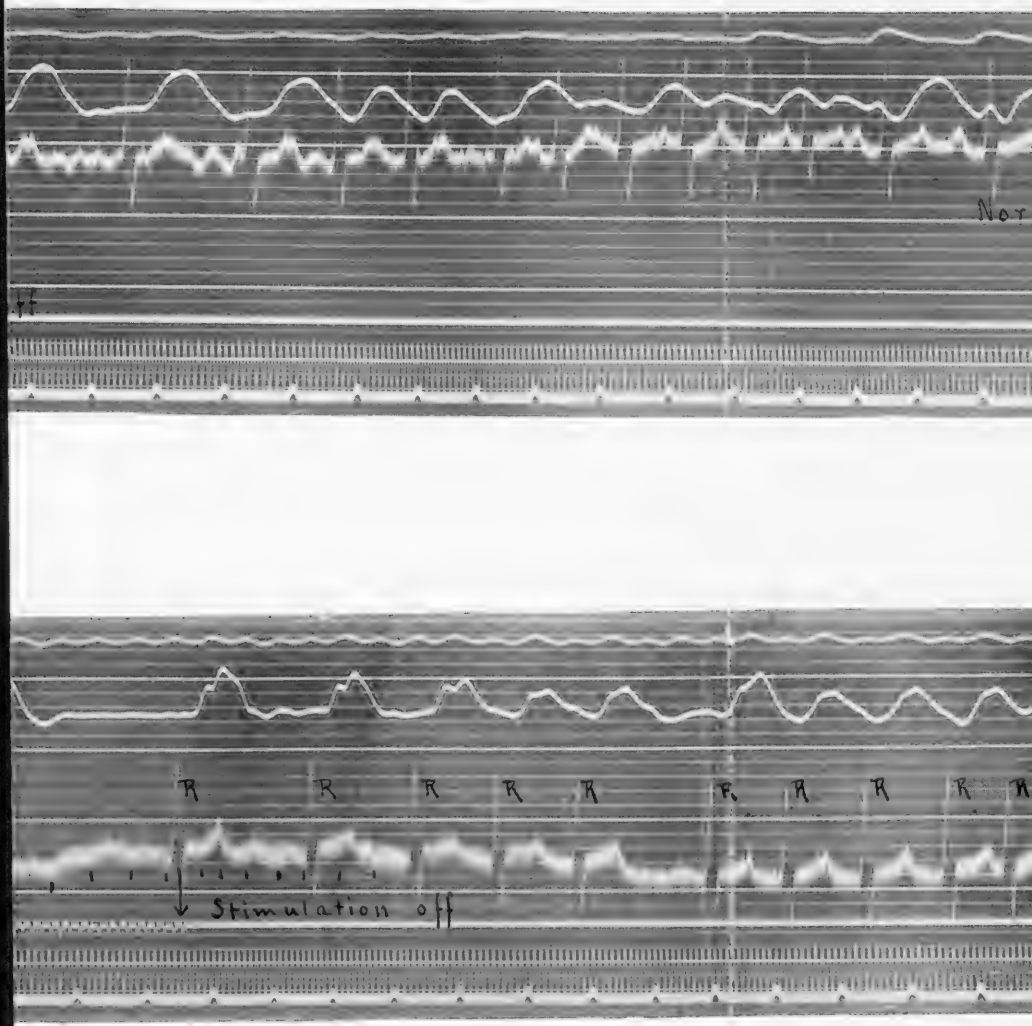


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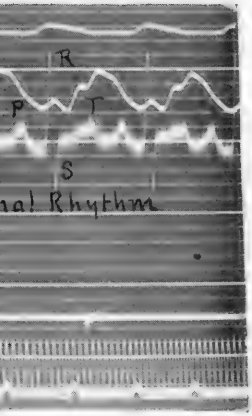
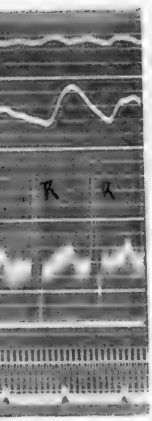
PLATE 76.



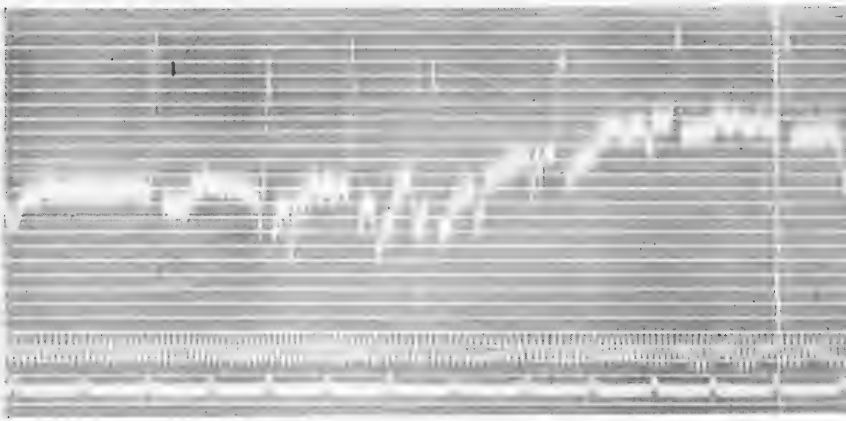


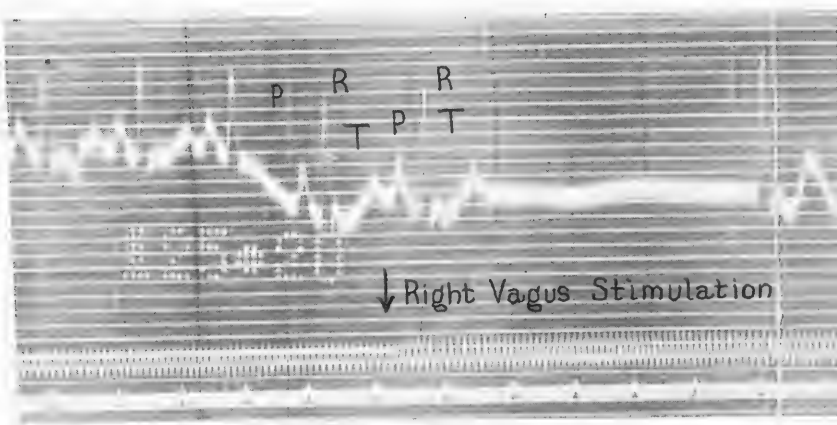
son : Influence of Vagus Nerves.)

PLATE 77.

FIG. 14. Experiment 25. Normal beat resumed 4 seconds after the end of right vagus stimulation of 5.4 seconds' duration. Auricular tachycardia had been present 24 minutes previous to the onset of right vagus stimulation.

FIG. 15. Experiment 25. Auricular tachycardia set up by right vagus stimulation of 3 seconds' duration. Tachycardia began during vagus stimulation, 1.2 seconds after the stimulation was begun.




P
R
T
P
R
T
↓Right Vagus Stimulation

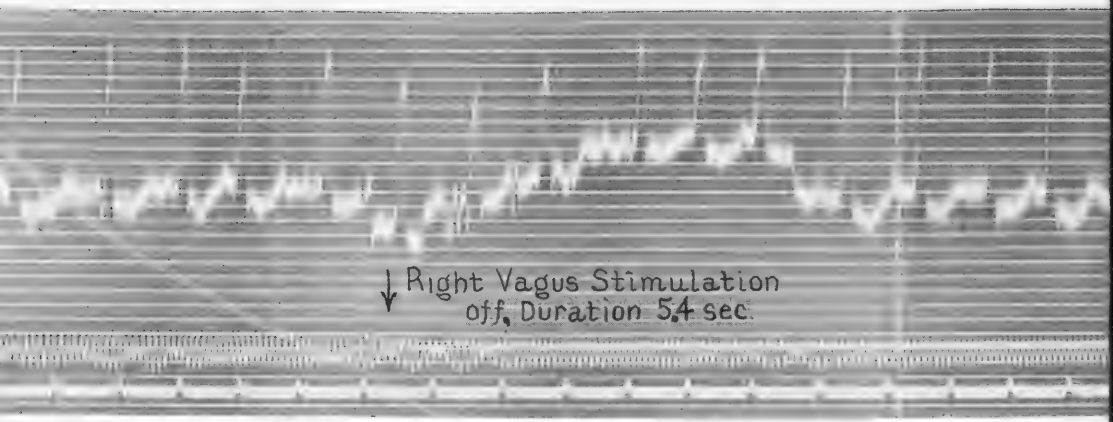

Right Vagus Stimulation
off, Duration 5.4 sec.

FIG. 14.

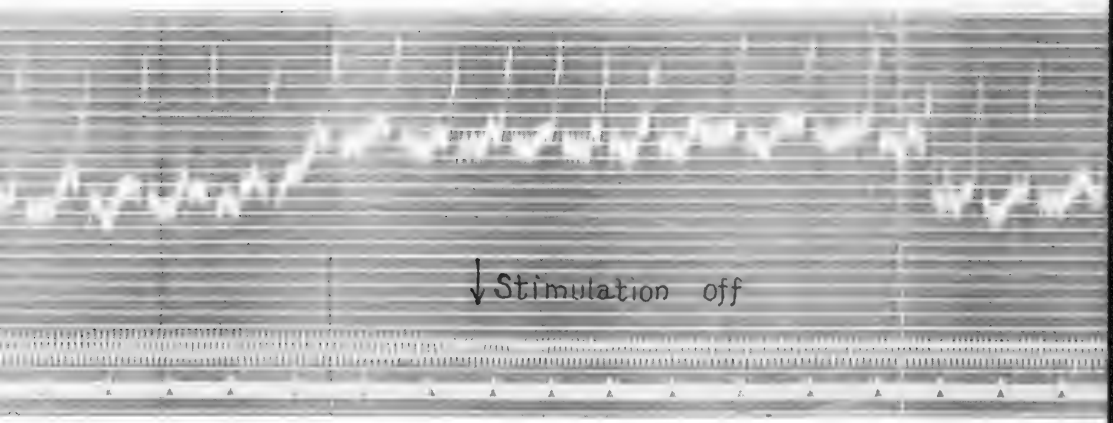
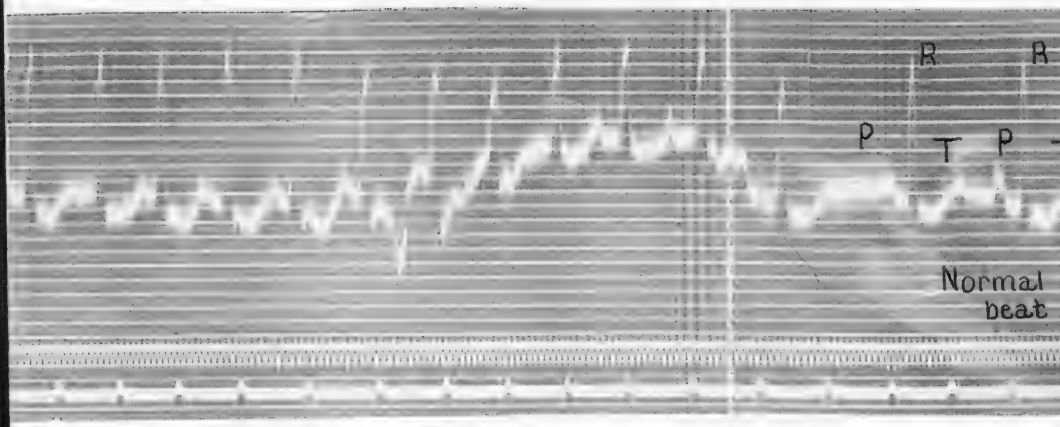
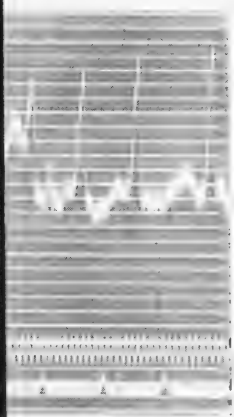

Stimulation off

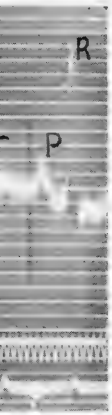
FIG. 15.





(Robinson : Influence of

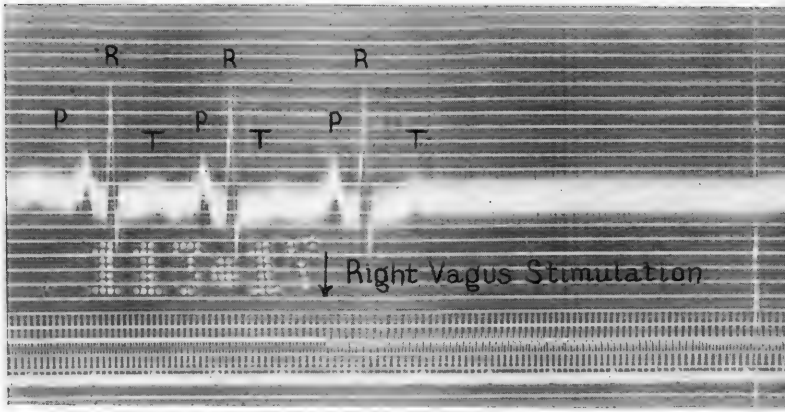
PLATE 77.

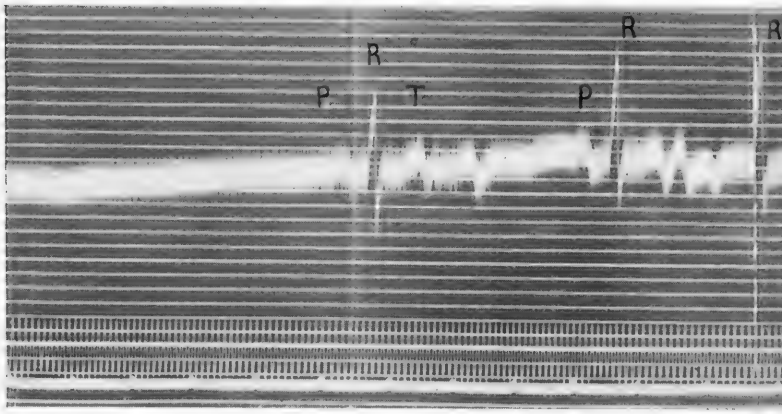


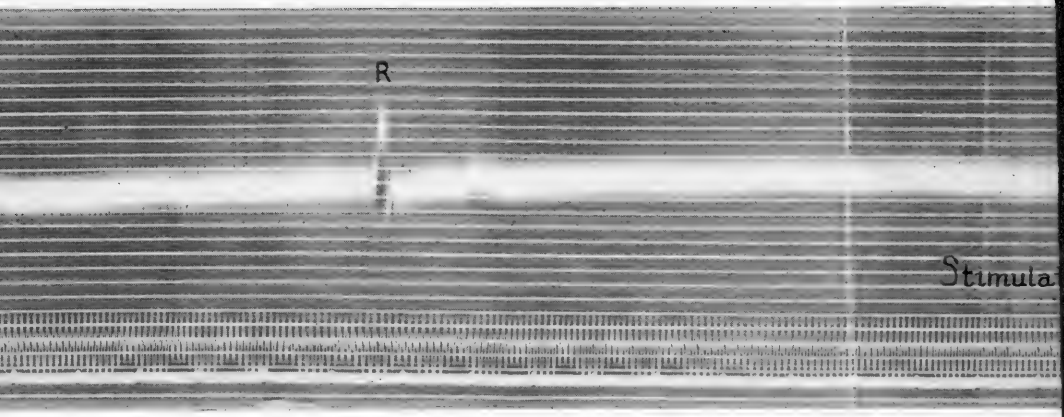
Vagus Nerves.)

PLATE 78.

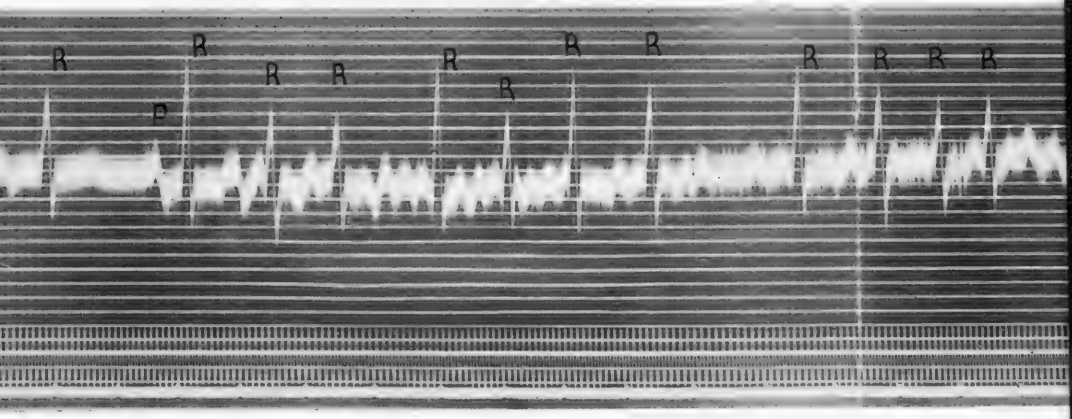
FIG. 16, *a* and *b*. Experiment 13. Auricular tachycardia set up by right vagus stimulation of 5 seconds' duration. Tachycardia began 3 seconds after the end of vagus stimulation. 16 *b* is a direct continuation of 16 *a*.


R
R
R
P
T
P
T
P
T
Right Vagus Stimulation


R
R
R
P
T
P



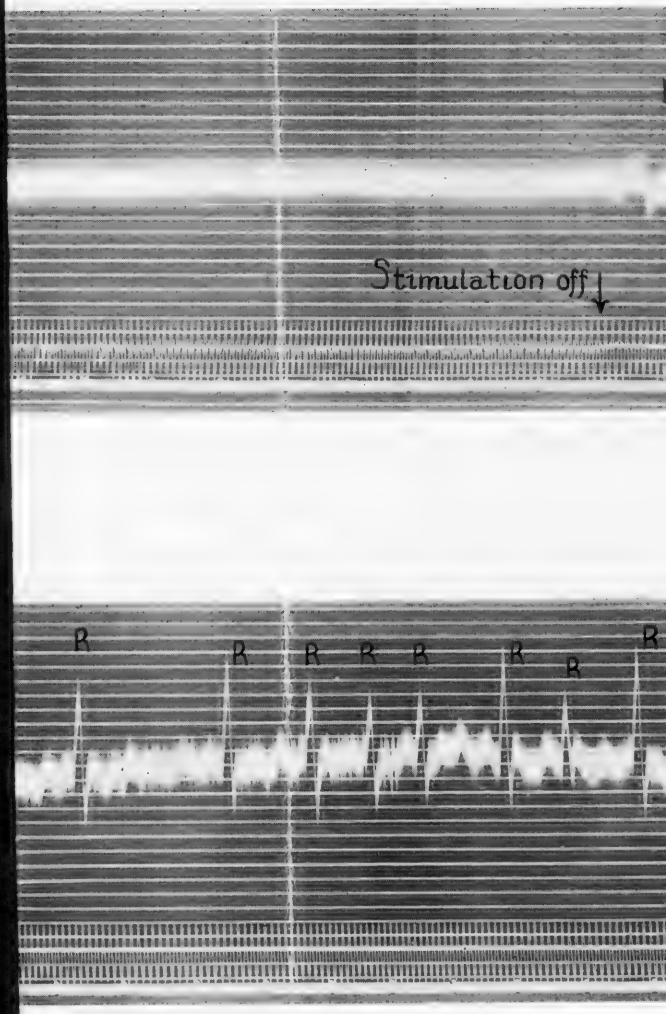
a.



b.

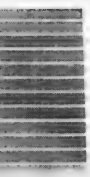
FIG. 16

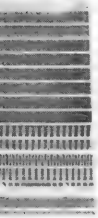
(Robinson



(Robinson : Influence of Vagus

ATE 78.





erves.)

established in hearts otherwise refractory by vagus stimulation of short duration following the faradization. Vagus stimulation usually holds the auricles in the abnormal activity set up by faradization as long as it is continued in hearts in which, without vagus stimulation, the sequential beat always returns as soon as faradization is stopped. The right vagus is more effectual in this respect than the left. In some hearts vagus stimulation alone is capable of initiating the same abnormal auricular activity which is caused by auricular faradization. The normal sequential beat is often restored by vagus stimulation. It replaces the abnormal auricular activity not during, but a few seconds after, the termination of vagus stimulation. Left vagus stimulation is somewhat more effectual in producing this result than right vagus stimulation.

BIBLIOGRAPHY.

1. Knoll, P., *Arch. f. d. ges. Physiol.*, 1897, lxvii, 587.
2. Hewlett, A. W., *Heart*, 1910–11, ii, 107.
3. Winterberg, *Arch. f. d. ges. Physiol.*, 1908, cxxii, 361.
4. Winterberg, *Arch. f. d. ges. Physiol.*, 1907, cxvii, 223.
5. Winterberg, H., *Ztschr. f. exper. Path. u. Therap.*, 1907, iv, 636.
6. Cushny, A. R., *Am. Jour. Med. Sc.*, 1911, cxli, 826.
7. McWilliams, J. A., *Jour. Physiol.*, 1887, viii, 296.
8. Fischel, R., *Arch. f. exper. Path. u. Pharmakol.*, 1897, xxxviii, 228.
9. Philips, F., *Arch. internat. de physiol.*, 1904–05, ii, 271.
10. Hirschfelder, A. D., *Bull. Johns Hopkins Hosp.*, 1908, xix, 322.
11. Garrey, W. E., *Am. Jour. Physiol.*, 1908, xxi, 283.
12. Lewis, T., *Heart*, 1909–10, i, 306.
13. Rothberger and Winterberg, *Arch. f. d. ges. Physiol.*, 1910, cxxxi, 387.
14. Kronecker, H., and Spillata, F., *Arch. internat. de physiol.*, 1904–05, ii, 223.
15. Cohn, A. E., *Jour. Exper. Med.*, 1912, xvi, 732.
16. Robinson, G. C., and Draper, G., *Jour. Exper. Med.*, 1911, xiv, 217.

FURTHER STUDIES ON THE CHEMISTRY OF BLOOD SERUM.*

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(From the Laboratory of Physiological Chemistry of the Pathological Department of Mount Sinai Hospital, New York.)

In a recent publication[1] the author recorded the results of a chemical analysis of blood sera from different diseased conditions. It was observed that the proteins of the serum are subject to extensive variations, and in certain diseases the globulin fraction in the serum is greatly increased. In the serum of cases of chronic parenchymatous nephritis the increase in the globulin content is most pronounced, and the globulin may constitute nearly all of the protein present. On the other hand, the protein fractions of sera from cases of chronic interstitial nephritis present, in their quantitative relations, little or no change from the normal. In certain cases of the latter group, there appears to be a tendency for the globulin in the serum to fall below the normal percentage.

For reasons which will be given in a future publication the author is of the opinion that the changes occurring in the protein composition of blood serum in different diseases are not accidental, but are the results of well defined influences acting upon the blood and its serum. In contrasting the findings in the sera of patients with chronic interstitial nephritis and with chronic parenchymatous nephritis, the conclusion is reached that the two diseases are genetically different in respect to the blood, and that the change in the protein composition of the blood serum plays a direct part in the production of some of the clinical manifestations of the two types of renal disease. The composition of the urine and the blood serum (the chlorides and nitrogenous waste products) in chronic interstitial nephritis depends upon the degree of impairment of the renal function; whereas in the chronic parenchymatous nephritis the chemistry

* This work was done under tenure of the George Blumenthal Jr. Fellowship in Pathology. Received for publication, January 21, 1913.

[1] Epstein, A. A., *Jour. Exper. Med.*, 1912, xvi, 719.

of the urine, the retention of fluid and salt in the body, and in a large measure the character of the disease in the kidneys, are due to changes occurring in the blood.

Since his last publication on the subject, the author has had the opportunity of examining a number of other blood sera from cases of genito-urinary disease. The particular object aimed at in the study of these cases was to ascertain to what extent localized renal affections react upon the composition of the blood, especially upon the protein content of the serum.

The cases studied are divisible into three groups. The first of these is comprised of minor surgical cases which may be regarded as normal. The second group consists of cases of hypertrophy of the prostate gland. Apart from the chronic interstitial nephritis found in many cases, this group is of interest because of the alleged effect upon the renal pathology and function of prostatic hypertrophy and the consequent retention of urine. The third series contains cases of localized renal affections, such as hydronephrosis, pyonephrosis, pyelonephritis, and nephrolithiasis. This group is important because the disease concerns the kidneys alone. Whatever changes arise in the composition of the blood can be definitely ascribed to disease of the kidneys.

Of the substances determined in the sera, mention will be made only of the total protein, of the incoagulable and non-protein nitrogen, and of the albumin and globulin contents.

The seven sera from the group of normal cases (table I) present values for the different substances which are in keeping with standard normal figures. The total protein in these sera ranges from 6.5 to 8.2 gm. per 100 c.c. of serum. The average of these figures amounts to 7.3 gm. The albumin present in all these sera is greater in amount than the globulin, which ranges from 2.1 to 3.2 gm. per 100 c.c. of serum, averaging 2.7 gm. per 100 c.c., or 37 per cent. of the total protein. In three sera the globulin content is somewhat higher than the average figure given; but in none does it rise above the quantity of albumin.

The incoagulable and non-protein nitrogen in this group of sera is variable, ranging from 0.055 to 0.173 gm. of nitrogen per 100 c.c. of serum. This constituent, even in normal serum, is more variable than the relation of the albumin and globulin to the total protein. In two of the cases (M. C. and H. G.) duplicate analyses of the sera were made at intervals of a few days. The results are of interest in showing the variability of the incoagulable nitrogen and the constancy of the protein constituents of the serum.

TABLE I.[2]

Normal Cases.

Case.	Date.	Diagnosis.	Total protein.	Incoagulable nitrogen.	Albumin.	Globulin.	Per cent. of globulin.
M. C.	Nov. 21	Varicocele	7.169	0.070	4.544	2.625	36.6
M. C.	Nov. 24	Varicocele	7.175	0.057	4.744	2.431	34.0
S. L.	Nov. 7	Varicocele	7.319	0.094	4.800	2.519	34.4
H. S.	Oct. 1	Fistula	7.094	0.055	4.169	2.925	41.2
A. S.	Oct. 8	Perineal sinus	8.190	0.090	5.134	3.056	37.3
H. G.	Oct. 4	Hemorrhoids	7.675	0.072	4.450	3.225	42.0
H. G.	Sept. 29	Hemorrhoids	7.619	0.076			
J. A.	Oct. 7	Orchitis	6.969	0.107	4.844	2.125	30.5
S. S.	Sept. 15	Varicocele	6.481	0.173	2.531	2.950	45.7
		Average.....	7.300		4.404	2.72	37.7

TABLE II.

Prostatic Cases.

Case.	Date.	Diagnosis.	Total protein.	Incoagulable nitrogen.	Albumin.	Globulin.	Per cent. of globulin.
I. S.	Oct. 17	Hypertrophy	6.963	0.056	4.600	2.3637	33.9
H. S.	Oct. 29	Hypertrophy	7.294	0.079	4.541	2.753	37.7
I. Z.	Sept. 4	Hypertrophy	7.550	0.257	2.225	4.325	57.2
E. P.	Sept. 4	Hypertrophy	6.938	0.227	3.096	3.842	55.3
I. S.		Hypertrophy	5.887	0.114	2.900	2.917	49.5
S. G.	Nov. 19	Hypertrophy	6.894	0.067	3.969	2.925	47.7
A. W.	Nov. 25	Hypertrophy	7.787		4.510	3.275	41.4
A. L.	Oct. 4	Hypertrophy	7.531	0.055	4.194	3.337	44.3
A. L.	Oct. 29	Hypertrophy	7.469	0.080	3.869	3.600	48.0
M. R.	Oct. 8	Hypertrophy	7.225	0.154	3.600	3.625	49.0
M. R.	Oct. 11	Hypertrophy	6.750	0.125	3.338	3.412	51.0
		Average.....	?	?	?	?	41.6

[2] In tables I, II, and III the values are given in grams per 100 c.c. of serum.

Bladder Tumor.

M. W.	Sept. 22	Papillary carcinoma of bladder	6.756		3.181	3.575	
M. W.	Oct. 8	Papillary carcinoma of bladder	6.719		3.407	3.312	
H. P.	Oct. 15	Carcinoma of bladder and prostate	7.938	0.060	4.776	3.162	

In the second group (table II) eleven sera are from cases of prostatic hypertrophy, one is from a case of carcinoma of the bladder and prostate, and one from a papillary carcinoma of the bladder The protein content of this group of sera is almost normal in amount. The figures range from 5.887 to 7.551 gm. per 100 c.c. of serum. Three of the sera (from cases I. S., H. S., and H. P., table II) present also normal relations of the serum proteins. In the remaining sera the globulin content is above the normal amount, but the increase in the percentage of this protein is moderate.

Clinically all the cases of this group showed marked vascular and pulmonary changes. In two of the cases (E. P. and I. S.) there was a pronounced emphysema of the lungs, and in another (I. Z.) a diffuse purulent bronchitis was also present. Attention is called particularly to these clinical features, because, as pointed out in the previous communication,[3] the globulin content in the serum of such cases is usually above the normal amount. In the sera of the cases presented the globulin content averages 41.6 per cent. This moderate increase is not to be attributed to any alteration of the kidneys, but rather to the other conditions accompanying the renal changes; namely, emphysema, bronchitis, etc.

Duplicate analyses were made on the sera from two of the above cases (A. L. and M. R.). In one instance (A. L.) the blood specimens were withdrawn at an interval of twenty-five days; in the other case (M. R.) three days elapsed between the removal of the two specimens of blood. The findings show a slight fluctuation in the protein content of the sera.

The variation in the amount of incoagulable nitrogen of the sera of these cases is very great, but when compared with the urinary findings in the corresponding cases, it appears that the amount of incoagulable nitrogen varies with the degree of impairment of the renal function.[4]

The two cases of neoplasm of the bladder (M. W. and H. P.) present no noteworthy features. The serum of one of these (M. W.) was examined on two occasions separated by an interval of fourteen days; the results of the second examination do not differ from those of the first.

Of the group of six cases of localized renal affections (table III), twelve serum examinations were made. The findings illustrate the

[3] Epstein, A. A., *loc. cit.*

[4] The deductions drawn concerning the renal activity are based upon functional tests, the results of which will appear in a publication by Dr. Leo Buerger, Dr. Lautman, and the author.

point in question; namely, that disease of the kidneys proper does not affect the composition of the serum proteins.

TABLE III.

Localized Renal Affections.

Case.	Date.	Diagnosis.	Total protein.	Incoagulable nitrogen.	Albumin.	Globulin.	Per cent. of globulin.
L. G.	Sept. 10	Coincident hydropyonephrosis; horseshoe kidney	6.875	0.165	3.163	3.712	53.8
G. T.	Sept. 30	Hypertrophy of prostate	8.506	0.109	4.881	3.625	42.6
G. T.	Oct. 2	Renal calculi	7.606	0.093	4.156	3.450	45.2
G. T.	Oct. 14	Pyonephrosis	6.513	0.063	3.326	3.187	48.6
G. T.	Nov. 21	Perinephritic abscess	6.812	0.060	2.962	3.850	56.5
M. G.	Oct. 7	Double kidney	7.344	0.265	4.207	3.137	42.6
M. G.	Oct. 11	Hydronephrosis	7.312	0.011	4.356	2.956	40.4
T. B.	Aug. 31	Pyelonephritis	8.250	0.112	4.991	3.269	39.6
B. H.	Aug. 24	Pyelonephritis	8.125	0.149	3.038	5.087	62.6
B. H.	Aug. 30	Pyelonephritis	8.144	0.335	3.458	5.686	69.8
C. D.	Dec. 20	Unilateral tuberculosis of		0.065			
C. D.	Jan. 11	the kidney	7.106	0.123	3.960	3.200	45.0

The cases referred to represent in the main pyogenic inflammations of the kidneys. To appreciate the net effect of such a process upon the blood serum it is necessary to consider, on the one hand, the effect of the infection upon the blood serum, and on the other, that of the destruction of kidney tissue and the impairment of the renal function.

Pathologically considered the infectious process in the kidneys is similar to that which may occur in any other part of the body. Its effect upon the chemical composition of the blood serum must be in all respects like that of a circumscribed infection or abscess formation in any other part of the body. As is well known, in all cases of pyogenic infection, but to a less extent in tuberculosis, the globulins in the blood serum are increased.[5] Thus we find in these cases that the globulin content in the serum is increased. The increase in the percentage relations of the globulin to the albumin and the total

[5] Erben, F., *Ztschr. f. klin. Med.*, 1903, l, 450.

protein appears to be proportionate to the duration and the extent of the infectious process. This inference, it seems, may be drawn from the results of the repeated examinations of the sera of two cases (B. H. and G. T.).

In the first case (B. H.) the blood serum was examined twice at an interval of six days. The clinical features in this case pointed to a rapidly progressing pyelonephritis. The chemical findings show that although the total protein in the serum remains constant, the globulin content rises from 62.6 per cent. to 69.8 per cent. The effect of the functional impairment arising from the destruction of renal tissue is manifested in the marked rise of incoagulable and non-protein nitrogen (from 0.149 to 0.335 gm. per 100 c.c. of serum).

In the second case (G. T.) the observations extended over a period of two months, and in all, four examinations of the blood serum were made. The clinical data in this case show that the disease progressed in two phases, one of which stands in relation to the protein composition of the serum, the other to the non-protein constituents of the serum. This patient had hypertrophy of the prostate, unilateral urethral and renal lithiasis, and pyonephrosis with a secondary perinephritic abscess, and in the course of the illness an extensive effusion on the chest developed. Disease in the second kidney was not demonstrable.

On the basis of what has been stated before, the results in this case show that the protein composition of the serum is affected by the pyogenic process, but is independent of the eliminatory function of the kidney; whereas the non-protein content is related to the activity of that organ.

It is seen from the figures in table III that the total protein in the serum falls progressively (8.506, 7.606, 6.513, and 6.812 grams per 100 c.c. of serum), and the amount of globulin remains constant; thus the percentage relation of the globulins to the total protein is progressively increased.

This result may be due to one or more of several causes: 1. A gradual increase in the globulin content with a progressive dilution of the blood (hydremia). The progressive loss of weight on the part of the patient argues against such an occurrence. 2. A greater loss of albumin than globulin in the purulent exudate discharged by the kidneys. The analyses of the urines made at different times show, however, that a greater loss of albumin in the pus could not account for the marked decrease in the total protein of the serum and the great rise in the percentage of globulin. 3. A greater loss of albumin in the serous effusion which occurred in the chest may

have contributed to the net result obtained. The total volume of fluid removed on aspiration of the chest amounted to 250 c.c. This fluid, unfortunately, was not submitted to chemical examination. 4. A greater destruction of serum albumin in the course of the protein metabolism in the body. This is a possible cause of the change observed in the composition of the serum, for the patient was kept for a long time on a diet rich in fats and carbohydrates but poor in proteins.

Table III shows that in this case the behavior of the non-protein nitrogenous substances is the inverse of that observed in the preceding case. The values drop gradually from 0.109 to 0.060 of a gram of nitrogen per 100 cubic centimeters of serum. This decrease becomes intelligible upon consideration of the clinical data. As stated above, the infectious process which originated in the kidney extended to the surrounding tissues, so that pus formation continued up to and beyond the time of the last serum examination. The effect of a pyogenic process upon the protein composition of serum has already been discussed. On the other hand, the renal function improved in consequence of surgical interference at the seat of disease. That an improvement in the renal function occurred was established by a study of the urine made at the same time that the different specimens of blood were examined. The fact that the patient was kept persistently on a low protein and high fat and carbohydrate diet may also have helped to reduce the non-protein nitrogenous bodies in the serum.

A third case of the same series (M. G.) shows even more strikingly the relation of the renal function to the non-protein composition of the serum, and the total lack of such relationship to the protein constituents. The two examinations of the blood serum were made four days apart; the first prior to, and the second after an operation upon the kidney. The results show that the protein content together with the relative amounts of albumin and globulin are within the normal range and remain constant. The non-protein nitrogen drops from 0.265 to 0.110 of a gram per 100 c.c. of serum. This result is in accord with the clinical developments of the case.

We have thus an indication of the twofold character of the chemical changes in the serum, resulting from localized disease of the

kidneys. The changes brought about by the infectious process affect the protein composition of the serum, whereas that which interferes with the renal function affects the nitrogenous waste products.

Another instance of the effect of the interference with the renal function upon the non-protein composition of the blood serum is found in the analysis of the serum of a case of unilateral renal tuberculosis (C. D.). In the first examination which was made prior to the operation upon the diseased kidney the amount of non-protein nitrogen in the serum was 0.065 of a gram per 100 c.c. After the removal of the diseased kidney this constituent of the serum rose to 0.123 gram per 100 c.c. Determinations of the urinary nitrogen made at the same time showed that the elimination of these substances after the operation was considerably less than that at the time of the first examination of the blood serum.

The other cases in this group show similar results. The nitrogenous waste products vary considerably in the different sera. The percentage of globulin is elevated in most of them, but this is attributable to the infectious process and not to any other cause with which the kidneys are functionally concerned. If we contrast the results obtained in these cases with those found in the sera of cases of chronic parenchymatous nephritis, reported in the communication already referred to, we find that in the case of localized renal affections the composition of the serum is entirely different. The globulin content in none of the latter cases reaches so high a level as that observed in the sera of parenchymatous nephritis.

SUMMARY.

The following conclusions may be drawn from the results obtained in this study.

1. In the minor surgical cases (considered normal) the chemical composition of the blood serum agrees, as far as its proteins are concerned, with the usual standard values. When examined at different times, the serum of such cases shows no variation in the total protein content or in its individual fractions. The incoagulable nitrogen, however, varies considerably in the total amount in the different cases, as well as in its percentage relations to the other constituents of the serum.

2. The cases of prostatic hypertrophy, with or without chronic interstitial nephritis, show no change from the normal in the character of the protein composition of the serum, nor in the ratio which the individual fractions bear to each other. On the other hand, the incoagulable and non-protein nitrogen of the serum show marked fluctuations, some of which correspond to the degree of functional deficiency of the kidneys.

3. In cases of localized infections of the kidneys, the changes in the blood serum are twofold. One concerns the proteins, and is traceable to the infection; and the other concerns the non-protein nitrogen, results from functional impairment, and varies with the amount of destruction of the kidney substance. Thus an increase in the globulins is observed in these sera similar to that occurring in infections in other localities; the non-protein content increases apparently in direct proportion to the degree of deficiency of the kidney, and becomes diminished when the function of the kidneys improves.

The author wishes to acknowledge his indebtedness to Dr. Leo Buerger for the privilege of studying the cases, and to Dr. Lautman, of the House Staff of Mount Sinai Hospital, for assistance in procuring the samples of blood.

STUDIES IN FERMENT ACTION.

VII. Toxic Split Products of Bacillus Typhosus.*

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The study of the various toxic substances liberated by or derivable from bacteria has stimulated a large amount of work since the discovery of the specific relations between particular microorganisms and definite pathological conditions, for it was early believed that the mere mechanical presence of bacterial bodies in the tissues of the infected individual could not be held responsible for the damage wrought. But notwithstanding the early recognition of the significance of these substances, and their continued study by many workers, there is much left undetermined in regard to the nature of bacterial toxins, and the field of specific serum therapy is yet largely before us.

Bacterial toxic substances are easily divided into different classes. In one class we can put the true soluble toxins of *Bacillus diphtheriæ, Bacillus tetani,* etc. These toxins are considered specific secretory products of certain bacterial cells, just as pepsin is a specific secretory product of certain animal cells. Animal organisms react to these toxins by producing specific counteracting bodies that neutralize the toxic bodies in definite multiple proportions, however high the multiple runs. In this type of infection a wide distribution of the causative organisms is not necessary for the production of general disturbances, since the toxic products are so easily separated from the bacterial bodies and so readily soluble.

The toxins or toxic substances of a second class of bacteria bear a very different relation to the bacterial bodies by which they are produced and the reaction products of inoculated animals are different from those produced by toxins of the first class. *Bacillus typhosus, Bacillus coli,* etc., produce toxins of this class. It is hardly

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conceivable that typhoid bacteria do not produce specific poisons that give rise to the symptom-complex which characterizes the disease as a definite clinical entity. Yet it has not been definitely determined that these bacteria manufacture and liberate a secretory toxin. Toxic substances have been obtained from typhoid bacteria, it is true, but a mechanical or chemical destruction of the bacterial bodies was necessary for their liberation. None of the various methods used to isolate toxins from *Bacillus typhosus* has given a product of any degree of purity or one which produced an antitoxin of great value as a protective or therapeutic agent. Therefore, any additional information concerning the nature and source of the toxic substances derivable from this class of organisms is of first importance, since it is only through a biological and chemical knowledge of these substances that we may hope to develop methods by means of which an effective curative agent can be produced.

HISTORICAL PART.

A complete review of the extensive literature concerning the toxins obtainable from typhoid bacilli will not be undertaken here. Only the publications bearing more or less directly upon the subject at hand will be referred to.

Pfeiffer (1) first advanced the opinion that typhoid toxins are liberated by a destruction of the bacterial bodies through the action of lytic substances in the body of the infected animal. He found no toxin in the filtrate of young cultures. His endotoxin produced bactericidal substances in treated animals. It was not destroyed by chloroform vapors, and it was thermolabile. Pfeiffer also believed that the endotoxin acted as an antigen in natural cases of the disease, giving rise to the antibodies in the blood of convalescents. Buchner (2) prepared poisonous bacterial proteins by evaporating emulsions of different bacteria to dryness, rubbing them up thoroughly in hot water, boiling for an hour in a reflux condenser, filtering through *Kieselguhr,* and condensing. The product thus obtained produced pus when placed in tubes under the skin of animals, fever and apathy in dogs, and very severe local and general reactions in tuberculous animals. Antigenic substances were prepared by Brieger (3) from three to four day cultures of typhoid bacilli by saturation with ammonium sulphate, allowing the mixture to stand in a dark place for from one to four days, collecting the precipitate and shaking it in water for several hours, and then passing the water through a Berkefeld filter. The final filtrate gave Millon's reaction, and produced agglutinins and precipitins in treated animals. Krehl and Matthes (4) in their work on the production of fever in animals by different protein fractions found that the deuteroalbumose fraction from various bacterial pro-

teins was the only portion which had any influence upon the temperature. These authors found that the deuteroalbumoses from fibrin, casein, muscle, egg albumen, etc., had the same properties as those from bacteria. Vaughan and his co-workers (5) washed bacterial bodies with alcohol, extracted them with ether, and pulverized them by thorough rubbing up in agate mortars. Bacteria thus prepared were boiled in a reflux condenser for an hour or more with several volumes of 2 per cent. sodium hydroxide in absolute alcohol. This procedure gave them a toxic and non-toxic portion; the former soluble and the latter non-soluble in absolute alcohol. The toxic portion was collected by evaporating the alcohol at a low temperature. It was largely precipitated by saturation with ammonium sulphate, and gave all the ordinary protein reactions, except that of Molisch. It was termed an alcohol soluble albumose. The lethal dose for guinea pigs varied from 8 to 60 mg. when given intraperitoneally. The animals suffered a slight initial rise and a subsequent greater fall of temperature, and became comatose some time before death. By this procedure toxic substances were obtained from pathogenic and non-pathogenic bacteria and proteins from other sources. The toxicity of a product was largely independent of the source of the protein from which it was derived, all producing practically the same symptoms and giving the same chemical reactions. Many other investigators, among whom are Macfayden (6), Besredka (7), Chantemesse (8), Sirotinin (9), and others have done more or less extensive work on the toxic substances of typhoid bacteria.

In regard to the symptoms and pathological changes noted in the experiments to be described later, it is necessary to state that these same conditions have been observed by Pearce and Eisenbrey (10) in a study of anaphylaxis in dogs. These authors used foreign serum to produce the anaphylactic state, and the symptoms and pathology as described by them are in every detail similar to the conditions observed in the present experiments. Schittenhelm and Weichardt (11) produced the same symptoms and pathological conditions in dogs by first injecting peptone or bacterial emulsions and by injecting egg albumen into dogs previously sensitized with the same substance. Rosenow and Arkin (12) obtained similar results by giving dogs intravenous injections of pneumococcic extracts. These authors observed that the lungs of the injected animals were especially affected, being hyperemic and hemorrhagic.

In considering the entire literature on this subject, one finds, with few exceptions, that the selection of the various methods used to obtain toxic substances from typhoid bacteria has been governed by the idea that a toxic body is stored in the innermost parts of the bacterial bodies, and sufficient mechanical division or pressure should liberate such a body. Even where digestion experiments were employed, the idea prevailed that such processes would break down the containing structures and thus liberate the stored up toxic body. Some workers, however, have believed that at least part of the toxic symptoms of natural typhoidal infections are due to split products of the native bacterial proteins, the hydrolysis of the proteins being caused by the action of ferments of the infected animal. Vaughan and his students have strongly advocated this idea.

In the present work the relation of the toxic substances obtained from *Bacillus typhosus* to the native bacterial proteins has been especially considered. Do these substances exist preformed in the bacteria, or are they formed by a cleavage of the protein constituents of the bacterial bodies? Can typhoid toxic products be isolated in any degree of chemical purity? And if processes more closely simulating those which operate under natural conditions are used to liberate these toxins, can antigenic agents be obtained that will produce clinically successful curative sera?

EXPERIMENTAL PART.

It has been observed by Schittenhelm and Weichardt that if certain quantities of typhoid bacteria are injected intravenously into dogs a definite set of symptoms develop a short time after the injection. The autopsy findings are also characteristic. The symptoms consist principally of an initial rise and subsequent fall of body temperature, vomiting, bloody diarrhea, coma, and finally death of the animal. At autopsy the abdominal organs are found to be greatly congested, the intestines being chiefly involved.

In order to observe the results of the intravenous injection of large quantities of typhoid cultures into dogs, to determine the effects of boiling the bacterial emulsion previous to injection, and to see if a solution of the bacteria by the addition of an alkali would in any way modify the results, the following three experiments were carried out.

Preparation of Bacterial Emulsions.[1]—The bacteria used in the experiments were obtained by growing a typical strain of *Bacillus typhosus* on plain agar for twenty-four hours, washing the bacteria with physiological salt solution, and centrifuging and washing them a second time. From the washed bacteria emulsions of approximately two and one half billions per c.c. were made in physiological salt solution. Such an emulsion will be referred to as a standard bacterial emulsion.

Experiment 1.[2]—2 c.c. of a standard emulsion of typhoid bacteria (five billion bacteria) were injected intravenously into a dog weighing ten pounds. Twenty minutes after the injection the animal became very sick and manifested, successively, vomiting, severe diarrhea which soon became exceedingly bloody,

[1] We wish to acknowledge our obligations to Dr. A. Goldfine for preparing a large proportion of the bacterial emulsions used in the experiments.

[2] All injections were given intravenously, and dogs were used as the experimental animals. The individual experiments were repeated many times with slight variations.

signs of exhaustion, labored breathing, stupor, and death. The animals died from two to six hours after the injection.

Autopsy.—A small amount of bloody fluid is found in the peritoneal cavity, the mesenteric vessels are hyperemic, the visceral peritoneum is rough and dull, subserosal hemorrhages are scattered over the small intestine, the kidneys are dark and congested, and the lumen of the small intestine contains much blood.

Experiment 2.—A standard bacterial emulsion was boiled for ten minutes over a free flame previous to injection. Otherwise the technique was the same as that of experiment 1. The symptoms and autopsy findings were not distinguishable from those obtained in the first experiment.

Experiment 3.—The bacteria of a standard emulsion were put into solution by the addition of 10 per cent. sodium hydroxide, neutralized with hydrochloric acid, and injected. The results were the same as those in experiments 1 and 2.

The results of the experiments show that a definite amount of typhoid bacterial bodies produces characteristic results when injected intravenously into a dog, that the effect of the injection is not appreciably modified by boiling the bacterial emulsion before the injection, and that a complete solution of the bacterial bodies with an alkali does not destroy the toxic properties. Therefore, it is evident that the toxic substance with which we are here dealing is bound up in the bacteria, not being removed by repeated washings, and, further, that we are apparently not dealing with a secretory toxin of any kind since such toxins have been found to be more or less highly thermolabile.

This evidence that the toxic substance that causes the symptoms and pathological conditions described in the above experiments is not a soluble, secretory toxin of the nature of diphtheria or tetanus toxin, and that its association with the bacterial bodies is an intimate one, led us to investigate the exact nature of the relation between the bacterial bodies and the toxic substance in question. As already stated, the idea prevails that a toxic body is stored in the structure of the bacterial bodies. To determine the relation of this toxic body to the coagulable proteins of freshly washed whole bacteria and bacteria put into solution by sodium hydroxide, experiments 4 and 5 were performed.

Experiment 4.—One or two drops of 1 per cent. alcoholic solution of phenolphthalein were added to about 100 c.c. of a standard bacterial emulsion and the mixture made definitely alkaline with 10 per cent. sodium hydroxide. After bringing this to the boiling point over a free flame, 5 per cent. acetic acid in 10 per cent. sodium chloride was added until no more precipitate formed.

The boiling was continued for a few minutes and the mixture was filtered through kaolin while hot and then passed through a Berkefeld filter. The filtrate was neutralized with sodium hydroxide and allowed to stand for several hours to permit the precipitation of any acid albuminates which might have been formed by the presence of the acetic acid used in the precipitation. The neutral solution was again passed through a Berkefeld filter. Intravenous injections of from 10 c.c. to 15 c.c. of this filtrate were without toxic effects upon the injected animals.

Experiment 5.—The bacteria of a standard emulsion were put into solution by adding sodium hydroxide and then precipitated and filtered as in the preceding experiment. The results of injection of this filtrate into dogs were the same as those of experiment 4.

The experiments show that a removal of the coagulable proteins from a young, freshly washed bacterial emulsion gives a comparatively non-toxic filtrate. The same results obtain even after a complete solution of the bacterial bodies before the removal of the coagulable constituents. Hence the toxic substance is either derived from the precipitable proteins or its association with them is such that it is mechanically carried down with the precipitate.

The mechanical and chemical methods used by many investigators to obtain toxic substances from typhoid bacteria are certainly different from any processes that could possibly occur in natural cases of the disease. This may explain, in part, the negative results of the active and passive immunization experiments with derivatives of these bacteria. In order to obtain a toxic product by processes which are more closely related to those that occur in nature, the following experiments were carried out.

Experiment 6. Leucocytic Ferment.—Inflammatory pus containing a high percentage of polymorphonuclear leucocytes was received from the operating rooms of the hospital immediately after removal from patients. After three washings with physiological salt solution, the cells were washed with alcohol and dried with ether. Thus prepared, the ferment can be kept in stock for several months without material deterioration. The ferment was prepared for use by extracting definite amounts of the dried cells with a weak sodium carbonate solution and centrifuging off the undissolved portion. The clear, supernatant fluid was used as the digesting agent, and a solution 0.3 c.c. of which would digest 2 c.c. of a 0.1 per cent. casein solution beyond the coagulable stage was termed a standard ferment solution. Jobling and Strouse (13) found that a leucocytic ferment prepared in this way contained no ereptic element and, therefore, left the zymolyte largely in the proteose stage.

To 25 c.c. of a standard bacterial emulsion were added 5 c.c. of a standard ferment solution. About 2 c.c. of toluol or chloroform were added as a pre-

servative, and the mixture was kept at 37° C. for five days. The digested material was now freed of coagulable proteins and acid albuminates, as described in previous experiments. The filtrate of this digested mixture was fatal to dogs in amounts varying from 1 to 2 c.c. The animals died from two to six hours after injection. The symptoms and pathological conditions were identical with those obtained by using the whole bacteria.

Experiment 7.—A bacterial emulsion was dissolved with 10 per cent. sodium hydroxide, neutralized with hydrochloric acid, and then digested, like the emulsion in the preceding experiment. After the coagulable proteins and acid albuminates were removed, the filtrate was as toxic as that obtained in experiment 6.

Experiments 6 and 7 show that the toxic substance of the bacterial bodies is not removed by an acid and heat precipitation after the emulsion has been exposed to the action of leucoprotease. Previous experiments showed that precipitation of the coagulable proteins removes the toxic substance from a fresh emulsion or from an emulsion put into solution with an alkali. Two possibilities confront us here: (1) a toxic body was liberated by the destruction of a containing structure, or (2) a toxic substance was formed from the coagulable proteins by a hydrolysis effected by the leucoprotease. The results of experiments yet to be described make the latter possibility the more plausible one.

To get a more accurate knowledge of the rapidity of the formation of the non-coagulable toxic substance, to eliminate the possibility of the mere presence of the ferment being responsible for the toxicity of the filtrate of the digested mixtures, and to determine whether continued digestion would destroy the toxic properties of a mixture, the following experiment was carried out.

Experiment 8.—A large quantity of a digestive mixture of bacterial emulsion and ferment was prepared. The emulsion and ferment solution were in the same proportion as those of the previous digestion experiments. A test specimen was removed immediately after the mixture was prepared, the coagulable proteins and acid albuminates were removed in the usual way, and the filtrate was tested for toxicity. Only slight symptoms were manifested in animals receiving from 10 c.c. to 15 c.c. of the filtrate. The mixture was kept at 37° C., and a specimen removed and tested at forty-eight hour intervals. The specimen removed after forty-eight hours was much more toxic than the first one; 10 to 12 c.c. being the lethal dose. Each succeeding preparation was more toxic until the fifth. From 1 to 3 c.c. of the four day specimen killed the animals injected. The toxicity gradually diminished, the twenty day test specimen being only slightly toxic.

These results show that the mere presence of the ferment solution is not responsible for the toxicity of the filtrate, that interaction of

the ferment and bacterial substance is necessary to change the toxic substance from a coagulable to a non-coagulable condition, and that continued digestion destroys the toxic substance.

To sum up: Freshly washed, unheated typhoid bacilli intravenously injected into dogs cause the development of definite symptoms as early as twenty minutes after the injection. Boiling for ten minutes does not destroy the toxic effects of a freshly washed bacterial emulsion. Complete solution of the bacteria of a fresh emulsion does not prevent the removal of the toxic substance with the coagulable proteins. The action of leucoprotease splits the toxic substance to a non-coagulable state, the digested mixtures being toxic after removing the coagulable portion. The mere presence of the leucocytic ferment is not responsible for the toxicity of the filtrate from the digested mixture, and continued digestion destroys the toxicity of a previous toxic mixture.

From these observations it is concluded that the toxic properties of freshly washed typhoid bacteria are not entirely due to preformed secretory toxic bodies that are stored in the bacterial bodies, but that these properties are due largely to products formed by hydration of the bacterial proteins through the agency of ferments present in the circulation of the animal previous to the injection, or which become mobile subsequent to the entrance of the foreign bodies into the blood stream. Since leucocytic ferments can attack the bacterial proteins *in vitro,* it is possible that the leucocytes are a source of the ferments which are active in experimental and natural cases of intoxication with the whole bacteria.

Having determined that the toxic substances of the digested bacterial emulsions are no longer precipitated with heat and acid, but remain in the coagulable protein-free filtrate, we decided to determine more definitely the chemical nature of the toxic substances. To this end the following procedures were adopted.

Large quantities of standard bacterial emulsions were digested with leucoprotease, as in the previous digestion experiments. These digested mixtures were freed of coagulable proteins and acid albuminates in the usual way. The lethal dose of the digested bacterial filtrates for dogs was then determined by intravenous injection. The remaining portion of the toxic filtrate was saturated with neu-

tral, recrystallized ammonium sulphate and allowed to stand until the precipitate thus obtained had collected into coagula. The precipitate was then collected by centrifuging at high speed, dissolved in water and precipitated, and collected a second time. This sediment was termed the whole proteose fraction. After removing as much of the adhering sulphate solution as possible, it was dissolved in water and tested for toxicity by the usual method. The whole proteose fraction was found to be very toxic. It was not possible to compare in a strict quantitative way the toxicity of the proteose fraction with that of the filtrate from which it was derived, since an unavoidable loss always occurred in the precipitation and collection. However, in several instances a lethal dose of whole proteose was obtained from less than two lethal doses of bacterial filtrate.

In precipitating and collecting the proteoses all the ammonium sulphate was not removed by the centrifuging previous to its solution in water and a small amount was, therefore, injected into the circulation of the animals. To eliminate the possibility of the toxicity of the proteoses being increased by the presence of the sulphate, ten times the largest possible amount present was given to dogs intravenously. No noticeable symptoms followed the injections. Thus dialysis or other procedures to remove all traces of the sulphate before injecting the proteose solution were rendered unnecessary.

The supernatant fluid from the first saturation was also tested for toxicity after the sulphate was removed. It was first passed through a Berkefeld filter to remove all traces of precipitate. The sulphate was then precipitated with barium hydroxide, and any excess of the barium was removed with carbon dioxide. The filtrate was kept at incubator temperature for several hours to evaporate the ammonia that had been liberated by the action of the barium hydroxide upon the sulphate. Large quantities of this salt-free solution were injected into dogs without toxic effects.

Further fractionation of the toxic product was attempted. Solutions of the whole proteose fraction prepared as already described were treated with an equal volume of a saturated ammonium sulphate solution. The precipitate was removed as usual, dissolved in water, and precipitated a second time. The supernatant fluid from the first precipitation was saturated with ammonium sulphate crystals

and the precipitate removed. Both sediments were now dissolved in water and tested for toxicity. It was found that the primary proteoses (half saturation precipitate) possessed a toxicity almost equal to that of the whole proteose solution from which it was derived, the secondary proteoses (saturation precipitate) being relatively non-toxic. More exact chemical methods would probably give a complete separation of the proteoses into a toxic and non-toxic fraction.

GENERAL DISCUSSION.

The severity and regularity of the intestinal lesions produced by intravenous injections of typhoid bacteria, or toxic products derived from them, incline one to believe that a special affinity exists between these substances and the intestinal tissues, especially the lymphoid tissue. In fact many investigators of this subject have considered these lesions to be absolutely specific. In a recent publication Arima (14) describes in some detail the symptoms and pathological lesions produced by injecting the toxic products of typhoid bacteria into rabbits, and he concluded that these results are due to a specific chemical affinity between the toxin and the tissues affected. The intestinal symptoms and pathology produced by Arima's typhoid toxins are similar to Schittenhelm and Weichardt's enteritis anaphylactica and can be produced by injecting sensitized rabbits or dogs with a toxic dose of the sensitizing protein or by injecting them with a first dose of typhoid, colon, or other bacteria, and are, therefore, not peculiar to typhoid bacteria or toxins.

At first we were inclined to believe that the swollen, hyperemic, hemorrhagic condition of the Peyer's patches and mesenteric lymph glands was due to a specific action of the toxic filtrate from the digested typhoid bacteria. Further experiments showed that the same condition could be produced by injecting a similar filtrate from *Bacillus coli,* meningococci, or *Staphylococcus aureus.* In these experiments the acute action of non-specific toxic proteins overshadows the action of any specific toxin that may be present, and the pathological picture is a non-specific one. Hence it is evident that lesions peculiar to the toxic product used in these experiments and resembling those of natural typhoidal infections cannot be pro-

duced by an acute intoxication of the experimental animals. It is possible, however, that a chronic treatment would give different results.

As shown by Pearce and Eisenbrey (10) and Schittenhelm and Weichardt (11), anaphylaxis in dogs is characterized by a fall of the blood pressure, vomiting, and bloody diarrhea. It is noteworthy that these symptoms are also produced by a single intravenous injection of freshly washed emulsions of typhoid, colon, and other bacilli. If the coagulable nitrogen is removed from these fresh emulsions, the filtrate is without effect. On the other hand, the filtrate from a digested emulsion produces the same results as the whole bacteria. Hence the coagulable proteins, or some derivative of them, are necessary to produce the anaphylactic results. Remembering, also, that a single injection of egg albumen, casein, etc. does not produce anaphylactic symptoms, and that Abderhalden (15) has shown that the sera of animals sensitized with native proteins possess proteoclastic ferments not found in the sera of normal animals, some interesting points are suggested. If the sensitizing dose of egg albumen, etc. acts by calling forth a ferment capable of attacking the native protein, the protein constituents of bacterial bodies are attacked by ferments incapable of acting upon the proteins ordinarily used in animal experimentation, since a sensitizing dose is not required. This biological difference between bacterial and other proteins may be due to the action of antiferments or to a denaturization of the proteins by the methods used in their isolation and purification. It is conceivable that the stability of the molecules of living, growing protoplasm is quite different from that of protein substances which have gone through the process of precipitation, coagulation, or dehydration. The influence of such physical or chemical states upon the parenteral introduction of proteins into animals and upon the toxic symptoms of general infections deserves investigation.

The classification of the toxic substances derived from typhoid bacilli and the particular part that each plays in natural typhoidal infections remain unsettled questions. The preponderance of opinion seems to be in favor of a true extracellular toxin to which, under proper conditions, an antitoxin is produced, and a so called endo-

toxin. The part played by these toxins individually in the production of the general symptoms and the local and general pathological conditions has never been positively determined. The thermostability of the toxic substance obtained by us does not permit its being classed with the extracellular secretory toxins. Pfeiffer's endotoxin was highly thermolabile, and all bacterial endotoxins are considered to be more or less thermolabile, being destroyed at 70° C. and below. As is shown by the experimental procedures we have described, the toxic substance obtained by digesting typhoid bacilli with leucoprotease is not destroyed by a heat and acid precipitation of the coagulable proteins, and withstands boiling for ten minutes and longer. Hence its relation to the toxic substances described as extra- and intracellular toxins cannot be decided without further experimentation.

In a consideration of the part this toxic split product plays in natural cases of the disease, a field for speculation arises.[3] The toxic substances of a fresh emulsion of the bacteria being removed by precipitating the coagulable proteins and not being thus removed from a digested emulsion, they are apparently formed by a hydrolysis of the native proteins. Moreover, this split product produces the same symptoms and pathology as the whole bacteria, and a further digestion destroys its toxicity. Moreover, the primary proteoses possess a large part of the toxicity of a digested mixture, an extensive cleavage not being necessary to reach the toxic stage. This is in harmony with the rapidity of the development of toxic substances following an intravenous injection of the bacteria and proves the presence of ferments capable of causing the first cleavage. The severe toxic effects that follow are evidence of an accumulation of these toxic compounds and prove the absence of agents capable of causing a rapid cleavage to non-toxic combinations. If these inferences are correct, it may be possible to produce this lacking ferment by active and passive immunizations. Experiments to test this and other points concerning the immunization with bacterial split products are now under way.

[3] The fact that typhoid fever is a septicemia justifies still more our drawing inferences from the intravenous injection of the bacteria.

SUMMARY.

1. A single intravenous injection into dogs of a sufficient number of freshly washed typhoid bacteria produces the symptoms and pathology that characterize anaphylaxis in these animals.

2. These effects are not produced by the coagulable protein-free filtrate from a fresh emulsion, while a similar filtrate from an emulsion digested with leucoprotease is very potent, the toxic portion of the bacterial bodies being changed from a coagulable to a non-coagulable state.

3. The symptoms and pathology described are not specific, since they can be produced by substances other than typhoid toxins.

4. Digestion with leucoprotease furnishes a method of liberating toxic substances from typhoid bacteria resembling the processes of nature more closely than the methods heretofore used.

5. The toxic substances thus liberated are not destroyed by a heat and acid precipitation of the coagulable proteins, and are of the nature of primary proteoses.

BIBLIOGRAPHY.

1. Pfeiffer, R., *Deutsch. med. Wchnschr.*, 1894, xx, 898; Pfeiffer, R., and Kolle, W., *Ztschr. f. Hyg. u. Infectionskrankh.*, 1896, xxi, 203.
2. Buchner, H., *München. med. Wchnschr.*, 1891, xxxviii, 45, 841.
3. Brieger, L., *Deutsch. med. Wchnschr.*, 1902, xxviii, 477.
4. Krehl, L., *Arch. f. exper. Path. u. Pharmakol.*, 1895, xxv, 222; Krehl, L., and Matthes, M., *idem*, 1895, xxxvi, 437; Matthes, M., *Deutsch. Arch. f. klin. Med.*, 1895, liv, 39.
5. Vaughan, V. C., *Boston Med. and Surg. Jour.*, 1906, clv, 215, 243, 271.
6. Macfayden, A., and Rowland, S., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1901, xxx, 753; Macfayden, A., *idem*, 1906, xli, 266.
7. Besredka, A., *Ann. de l'Inst. Pasteur*, 1905, xix, 477.
8. Chantemesse, *Compt. rend. Soc. de biol.*, 1897, xlix, 96.
9. Sirotinin, W., *Ztschr. f. Hyg. u. Infectionskrankh.*, 1886, l, 465.
10. Pearce, R. M., and Eisenbrey, A. B., *Proc. Soc. Exper. Biol. and Med.*, 1909–10, vii, 30; *Jour. Infect. Dis.*, 1910, vii, 565.
11. Schittenhelm, A., and Weichardt, W., *München. med. Wchnschr.*, 1910, lvii, 1769; 1911, lviii, 841; *Deutsch. med. Wchnschr.*, 1911, xxxvii, 867; Schittenhelm, A., and Grisshammer, W., *Ztschr. f. exper. Path. u. Therap.*, 1911–12, x, 412; Schittenhelm, A., and Hartmann, F., *ibid.*, 448; Schittenhelm, A., and Weichardt, W., *idem*, 1912, xi, 69.
12. Rosenow, E. C., and Arkin, A., *Jour. Infect. Dis.*, 1912, xi, 480.
13. Jobling, J. W., and Strouse, S., *Jour. Exper. Med.*, 1912, xvi, 269.
14. Arima, R., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1912, lxiii, 424.
15. Abderhalden, E., *Ztschr. f. physiol. Chem.*, 1912, lxxxii, 108.

A TRANSPLANTABLE NEW GROWTH OF THE FOWL, PRODUCING CARTILAGE AND BONE.*

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PLATES 79 TO 83.

Neoplasms of the common fowl have been described by numerous authors, and include tumors of various classes. Up to the present time four transplantable tumors of the fowl have been reported, one of which is the growth here dealt with. Fujinimi and Inamato in 1910 reported at a meeting in Japan a myxosarcoma which they had succeeded in transferring to other fowls.[1] In the same year Rous[2] described the transplantation of a spindle-celled sarcoma which at times showed myxomatous tendencies. This tumor was highly malignant, usually leading to the death of the animal, and often producing widespread metastases. The tumor reported by Fujinimi and Inamato appears to have been not so malignant, though sometimes metastasizing and causing death. The tumor which forms the subject of the present paper is an osteochondrosarcoma and has been mentioned in an earlier note[3] from this laboratory, in which was also reported the fourth transplantable chicken tumor, a spindle-celled sarcoma. For purposes of reference the osteochondrosarcoma is known in this laboratory as Chicken Tumor VII. It has great interest from at least two points of view. Its cells regularly undergo a process of metaplasia and differentiation to form cartilage and bone; and the growth is transmissible, like the spindle-celled sarcoma of Rous, by an agent separable from the tumor tissue and filterable through Berkefeld filters.[4] The filtrate,

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[1] Fujinimi, A., and Inamato, K., *Verhandl. d. Jap. path. Gesellsch.*, 1911, 114.

[2] Rous, P., *Jour. Exper. Med.*, 1910, xii, 696; 1911, xiii, 397.

[3] Rous, P., Murphy, J. B., and Tytler, W. H., *Jour. Am. Med. Assn.*, 1912, lviii, 1682.

[4] Rous, P., Murphy, J. B., and Tytler, W. H., *Jour. Am. Med. Assn.*, 1912, lix, 1793.

when brought into contact with connective tissue under suitable circumstances, gives rise to the characteristic growth in which is laid down cartilage, followed often by bone.

DESCRIPTION OF THE ORIGINAL GROWTH.

The fowl bearing the original growth was obtained while yet alive from a dealer in poultry. It was a Plymouth Rock hen, in good condition, of approximately pure breed, and apparently about a year old. On the lower portion of the keel of the sternum was an irregularly spherical mass, so symmetrically disposed that the keel passed almost through its center. It measured 6.6 by 5.7 by 4 centimeters. The growth was smooth, nearly as hard as bone, and the skin over it was slightly stretched but not firmly attached (figure 1). A wedge-shaped piece of it was taken out under ether anesthesia. The tissue thus obtained was found to be firm, white, and fibrous, with some translucent areas suggesting cartilage. Small bits were at once implanted in the original fowl, by means of a trocar (Bashford needle). Two such grafts were placed in the pectoral muscles, and four subcutaneously, on each side.

An examination of the fowl thirty-six days later showed that the original mass had not grown, except perhaps at the edges of the recent incision. The gap left by the removal of tissue had persisted almost unchanged, and was filled with purulent material. At six of the ten inoculation sites small nodules were felt. The fowl was in poor condition, so it was killed, and bits of the tumor tissue were inoculated into twenty young, pure-bred Plymouth Rock hens, the trocar again being used. Two of these died of intercurrent disease, but in twelve of the eighteen that survived growths developed, appearing in general within thirty days. The propagation of the tumor has since been easy, and it is now in its seventh transplantation generation.

GENERAL MORPHOLOGY OF THE GROWTH.

The general morphology of the transplantation tumors does not differ from that of the original or spontaneous growth. The latter was removed at autopsy together with the lower half of the

sternum, and was cut transversely It was well encapsulated. In the gross specimen the sternal keel could be traced to the growth's center, but here it was lost in a mass of red, spongy, bony tissue which radiated from it a distance of one to one and one half centimeters (figure 2). Peripheral to this the tumor was hard and white, with fine strands of opaque, fibrous tissue separating more translucent, homogeneous areas. The secondary nodules, globular and well defined, were composed of the opaque, fibrous tissue. The fowl was emaciated. No metastases were found.

Microscopically the capsule of the original growth was found to consist of fibrous connective tissue containing isolated muscle bundles. The growth itself was made up of a zone of what may be termed prechondral tissue, enclosing and grading into a mass of hyaline cartilage through which ran the sternal keel. From this latter numerous irregular, bony trabeculæ radiated into the cartilage.

HISTOLOGICAL FINDINGS.

The outer, or prechondral, tumor tissue is an irregular connective tissue composed of sparsely scattered cells in a relatively large amount of fibrillar intercellular substance. The cells closely resemble normal connective tissue cells of the young fibroblast type, having large vesicular nuclei, some with two nucleolar masses and some showing amitotic division or nuclear budding. The cells lie, singly or in groups, in slit-like spaces, and in the groups are larger and more globular. Blood vessels are numerous, and their walls consist usually of endothelium only. The intercellular substance gives the staining reaction for collagen.

Deeper in are occasional islands in which the cells are polyhedral or globular, and lie in irregular spaces suggesting capsules, while the intercellular substance is here relatively homogeneous, though non-staining. These islands strongly suggest cartilage, and in fact all transitions are found between them and actual cartilaginous areas lying further toward the center of the tumor. The cartilage cells are globular, in well formed capsules, and the ground substance stains deep blue with hematoxylin, taking an intense blue at the capsule rims. With aniline blue the ground substance takes a light, diffuse stain and also shows numerous fine, deeply staining, collagen fibrils.

Near the center of the growth the cartilage undergoes a transition to a non-calcified, osteoid tissue, which itself undergoes calcification and partial resorption. It is in this way that the radiating bony trabeculæ already mentioned appear to have been formed. The deeply staining cartilage masses, at their inner border, shade into a zone with a non-staining, homogeneous ground substance, the cartilage cells at the same time showing various stages of transition to cells with the general morphology of bone corpuscles (figure 7). Still further

in, calcification is taking place with the formation of trellis-like figures, staining deep blue with hematoxylin, between the cells of the osteoid tissue. Into this calcified zone, which in most places is quite narrow, marrow spaces, with their blood vessels and osteoclasts, are advancing, and resorption is taking place. Thus are formed irregular bony trabeculæ. The intertrabecular marrow spaces contain a loose connective tissue, for the most part only sparsely cellular, but in places packed with cells that resemble fibroblasts. Occasionally small collections of granular leucocytes, mostly of the myelocyte type, are seen. Blood vessels are small and not numerous. Deep in the mass the keel of the sternum is recognizable as two roughly parallel bony plates (figure 4). The bony trabeculæ which radiate from the outer surface of these into the tumor tissue closely resemble the trabeculæ of the medullary cavity. The periosteum can be traced from the base of the sternum up to the lowest of these bony processes on each side, but here it is lost in tumor tissue. Toward its free edge the sternal keel is broken through in many places and here the newly formed bone is continuous with the medullary trabeculæ. The edges of the gaps show active erosion by osteoclasts.

The secondary subcutaneous and intramuscular nodules consist practically entirely of prechondral tissue of the sort described. This tissue is much more cellular than in the original tumor, the cells lying close together with but a small amount of intercellular substance. Many of them have two nucleoli, and some show amitotic division in progress, or, more rarely, a mitotic figure may be seen. At its margin the prechondral tissue extends out between the muscle bundles, while external to this there is, amid the muscle, a diffuse increase of connective tissue.

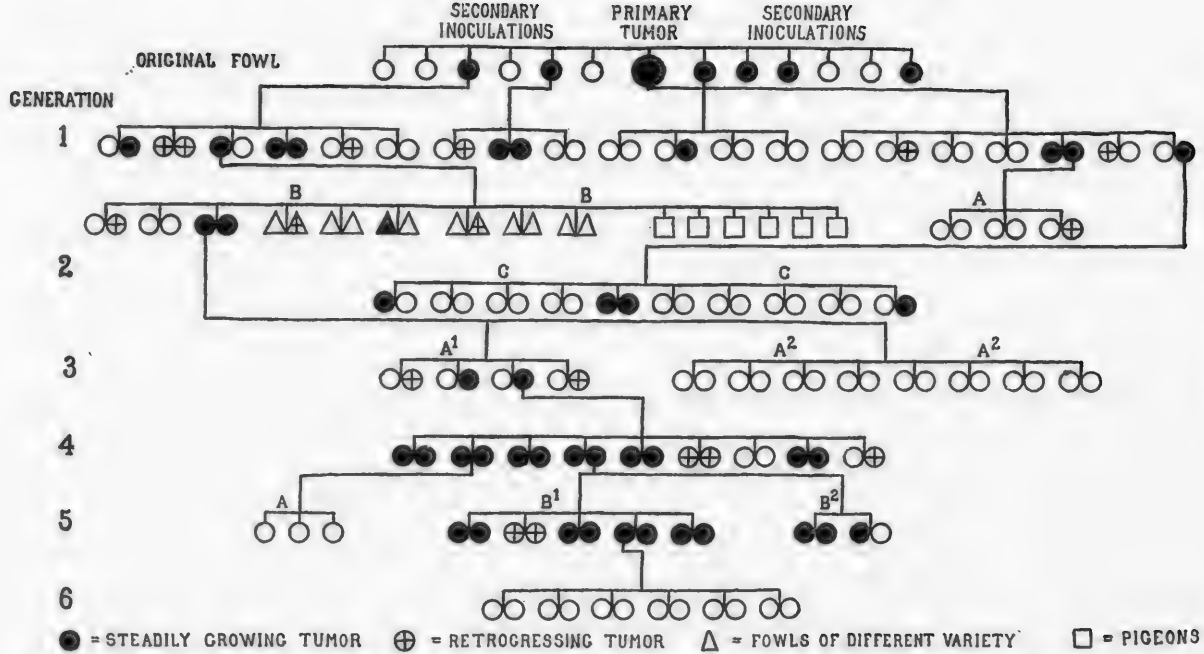
To recapitulate briefly: The outermost, youngest zone of the tumor is made up of cells of fibroblastic type scattered sparsely in a collagenous intercellular substance. In its deeper portions this tissue is undergoing a transformation to cartilage, its cells taking the character of cartilage cells, while the ground substance becomes homogeneous and basic staining. The original growth is well encapsulated, and seems nearly, if not quite, stationary. The secondary tumors have grown rapidly, are more cellular than the primary growth, and are invasive in tendency.

GENERAL RESULTS OF TRANSPLANTATION.

The growth has been propagated in series of chickens by transplanting small bits of the fresh, peripheral, neoplastic cartilage. In general, Plymouth Rock chickens have been used, and by means of a trocar the grafts have been placed deep in the pectoral muscles. In one series of four fowls prechondral tumor tissue was placed in one breast, in the other cartilage from the same tumor. Growths

TEXT-FIG. 1. Chart showing the results of implantations in the right and left pectoral regions of the twelve susceptible fowls of the first transplantation generation. The tumor growth is represented as a black band, the thickness of the band being that of the average diameter of the growth at any one time, and its length that of the number of days that the growth endured. The angular outlines of the bands are referable to the fact that they were plotted from measurements taken at considerable intervals. Rapid growth followed by slowing or retrogression is characteristic of Chicken Tumor VII.

From the figure it would appear that the tumors began to grow immediately on the implantation of the grafts. This was probably not the case, but a more correct chart cannot be made since the first examination did not take place until thirty days after the implantation. 809, 810, etc. = index number of the individual fowl. *R* and *L* = right and left pectoral regions.



TEXT-FIG. 2. The fate of the osteochondrosarcoma in the first five tumor generations. One negative series of the sixth generation is also figured.

developed in all four hosts, but only from the cartilage. The results of the transplantation from the original fowl are shown in text-figure 1, which also illustrates well the general behavior of all the propagated growths. The varying success of the tumor in its first five transplantation generations is shown in text-figure 2.

The percentage of successful inoculations from the primary growth was very high,—60 per cent. of the fowls yielding tumors and growths developing at 40 per cent. of all the inoculation sites. The first transplantation of spontaneous mouse tumors does not ordinarily yield nearly so high a proportion of growths. It is interesting to note that in Haaland's[5] series of seventy-eight spontaneous mouse tumors that were transplanted, the greatest average success was obtained with growths that on transplantation showed a general tendency to retrogress. In the case of Chicken Tumor VII this tendency is very marked.

Passage from host to host has scarcely increased the transplantability of the chicken tumor (text-figure 2). Furthermore, at present (the seventh tumor generation) it grows little if any more rapidly than when first transferred. But the prechondral tissue frequently infiltrates the surrounding muscle; and in one recent case a metastasis was found. For these reasons the tumor is best considered as an osteochondrosarcoma.

The development of Chicken Tumor VII after the implantation of a graft follows certain regular lines. The immediate fate of the implanted tissue has not yet been traced, but where it is placed in susceptible fowls there appears after ten days or two weeks a discrete, smooth, firm nodule which usually enlarges rapidly. It is ellipsoidal with its long axis in the direction of the muscle fibers. In one instance a mass four centimeters in diameter developed within thirty days after the implantation of a tumor bit not larger than four by one millimeters. There is often some invasion of the tissue surrounding such a nodule, but its growth is predominantly expansive in character and often it can be shelled out of its capsule, which at this time is usually well developed. Microscopically the tumor is found to consist of the peculiar prechondral tissue that

[5] Haaland, M., *Fourth Scientific Report of the Imperial Cancer Research Fund*, 1911, 1.

has been described in connection with the original growth. Cartilage is already forming at its center, as islands which gradually enlarge and merge into a mass that seems homogeneous to the naked eye, and as the mass grows larger degenerative changes occur at its center. With the gradual transformation to cartilage there takes place a slowing of the tumor's growth rate (text-figure 1), and after about fifty days nearly all the tumors become stationary, and many begin to retrogress. In some of the growing tumors, the proliferation of the prechondral rim and its transformation to cartilage slowly continue. If the host lives long enough ossification occurs within the cartilage and nearly the whole tumor may be converted into bone. The bony change takes place quite independently of whether the tumor is growing, stationary, or retrogressing, but in any event bone does not appear in the growth until the third or fourth month.

Instances in which this tumor has led to the death of the host are as yet few. Even when quite large it does not appear to cause ill health. At present there are in the laboratory numerous fowls which for many months have carried the growth in the breast as a stationary, retrogressing, or slowly growing lump of bony hardness. In the instances in which the tumor proved fatal the host has gradually emaciated and at length become comatose for a day or so before death. At autopsy no changes were found in the viscera other than those attendant upon emaciation and secondary anemia.

SPECIMEN PROTOCOLS.

A Progressively Growing Tumor.—Fowl 504, 7th generation A. In each breast of this fowl there was implanted a graft from No. 321, 6th generation A. In the right breast there soon developed a growth like a slightly flattened sphere, lying within the muscle, which increased rapidly in size up to the time of the fowl's death, sixty-seven days after the implantation. At autopsy the mass measured 6.4 by 5.6 by 4.5 cm. It projected sharply from the breast of the emaciated host. It was not attached to the sternum, but the pressure of its enlargement had arched inward the membrane stretching between two sternal ribs lying directly behind it. The growth was very firm and on section discrete, grayish white, of smooth, close texture, and translucent. Its center was honeycombed with blood channels, and showed at one point the old clot of a hemorrhage, at another a few bony spicules. Microscopically the growth consisted of hyaline cartilage with a thin rim of prechondral tissue. The central blood channels were walled only by endothelium. No metastases were found anywhere. It is doubtful if death was due to the tumor.

A Metastasizing Tumor.—Fowl 46, 2d generation C. A bit of tumor from No. 824, 1st generation A, was implanted in the muscle of each pectoral region of this fowl. Three weeks later a nodule about 1 cm. in diameter had developed at one site and after two months a similar growth appeared at the other. The tumors grew slowly and at length became stationary, remaining so for many weeks before the fowl's death which occurred from intercurrent causes seven months after the inoculation. At autopsy the tumors were found to be shaped like slightly flattened spheres. They measured 5 and 1.7 cm. respectively, in their greatest diameter, were discrete and stony hard, and consisted of a narrow, gristly rim of cartilage about a mass of spongy bone and red bone marrow.

On cutting away the sternum a characteristic tumor mass, 0.9 cm. in diameter, was disclosed, projecting sharply into the thoracic cavity and attached by a narrow base to the inner surface of the lowest right rib at the junction of its sternal and vertebral portions. Its situation was such that it could not be attributed to the primary implantation. It contained cartilage.

A Stationary, Bony Tumor.—Fowl 823, 1st generation A, and 6th generation A. This fowl was inoculated with two grafts of the original osteochondrosarcoma, and developed as a result one small tumor which was largely removed at operation, the remainder of it retrogressing. One hundred and five days after its disappearance a reinoculation was done, this time with material from No. 353, 5th generation B. A tumor developed which within a month reached a diameter of 2.5 cm., but then ceased to grow. Four months later, when the fowl was killed, it measured only 2 cm. across. At autopsy it proved to be a roughly spherical, bony nodule, with a smooth surface and a thin connective tissue capsule. It was made up of an outer shell or lamella of bone enclosing spongy bone and red marrow and a few islands of cartilage. The general health of the host had been good throughout.

HISTOLOGY OF THE METAPLASIA.

The formation of cartilage which takes place regularly in the transplanted growths (figures 5, 9, and 10) comes about in the same manner as in the original tumor. The islands of cartilage, at first isolated in prechondral tissue, gradually enlarge and become more numerous until to the naked eye their fusion appears complete. As a matter of fact, the microscope shows that they are still separated by thin strands of the prechondral tissue. In its interior the cartilage sooner or later undergoes degeneration, with loss of staining power and gradual death of the cells.

The change to bone also occurs in the same way as in the primary tumor. The cartilage undergoes a transformation to osteoid tissue, becomes calcified, and is actively eroded by osteoclasts to form trabeculæ between which red bone marrow appears (figure 6). There is thus formed an irregular mass of spongy bone. The

process, as in the primary tumor, seems to be one of progressive resorption, with no production of new bone except as is obtained by the continuous conversion of cartilage into a calcified, osteoid tissue. Osteoblasts appear to play no part in the bone formation.

The bone cells are somewhat irregular in morphology, but the majority closely resemble normal bone corpuscles, being small, flattened, sharp angled, dark staining cells, lying in clear cut spaces. The ground substance is dense and homogeneous. The marrow spaces show large blood spaces, walled with endothelium and distended with red blood cells. Most of the red cells are not peculiar, but many are more globular than the normal and have a bluish grey cytoplasm (after eosin and methylene-blue) and pale, rounded, vesicular nuclei. Occasionally a mitotic figure is seen in one of these cells.

In the intervascular tissue are seen numerous spindle cells of the type seen at the growing border of the tumor. These cells occur also in more globular form, many with few or no cytoplasmic processes, but with the same large vesicular nuclei and pale staining cytoplasm as the prechondral cells. Small numbers of cells occur, however, of the same size and shape as these globular cells, and with the same type of nucleus, but with a strongly basophilic cytoplasm which may contain occasional eosinophilic granules. The nuclei occasionally show mitosis. These cells closely resemble the strongly basophilic cells seen in the development of human blood, and designated as *Wanderzellen,* myeloblasts, etc., by various authors (Maximoff, Askanazy, Schridde, Naegeli, and others). With these cells occur mononuclear cells filled with eosinophile granules, and the ordinary polymorphonuclear eosinophile leucocytes. These four types of cells, the globular, pale staining connective tissue cell, the basophilic cell with few or no granules, the myelocyte, and the polymorphonuclear leucocyte, are often seen lying together in nest-like collections, the last two types always being in excess. The suggestion is very strong that the myelocyte type takes its origin from the basophilic cell, which in turn seems to be related to the globular connective tissue cell. Whether these latter take their origin from tumor cells, representing thus another metaplastic change, has yet to be determined.

The striking characteristic of the retrogressing neoplasms is the replacement of tumor by fibrous connective tissue which from its maturity and uniformity would appear to be derived from the host tissues. The tumor is separated by this into small, sharply defined nodules, which consist of cartilage, or, in the case of the more outlying ones, of dense collagenous tissue that is still living. At the margin of the mass are many good sized blood vessels, surrounded usually by numerous lymphocytes. In old, stationary growths cartilage and prechondral tissue may practically disappear leaving a rounded, bony nodule consisting of a smooth outer lamella, or bony shell, and a spongy interior of trabeculæ and red marrow.

INTRAVENOUS INOCULATION.

Three fowls of the 5th generation A received at the time of their original intramuscular inoculation an intravenous injection of a suspension of the finely ground tumor in Ringer solution. All fowls developed tumors in the breast muscle as a result of the inoculation at that site. Two of them showed at autopsy no visceral lesions. In the third fowl, No. 352, dying 136 days after the injection, there were found, in addition to the large, bony, pectoral growths, two tumor masses attached to the ovary, and each the size of a hen's egg, in which coils of small bowel were involved. There were also irregular disseminations of tumor tissue, up to 0.5 cm. in thickness, on the parietal and visceral surfaces of the peritoneum. Microscopically both the ovarian and the peritoneal growths showed characteristic prechondral tissue and cartilage. The ovarian tumors also showed extensive hemorrhage and necrosis. Whether these visceral growths resulted from the material injected intravenously, or were secondary to the pectoral tumors, cannot be definitely said. The relatively slight metaplastic changes observed in them favors the latter conjecture since it points to a recent origin for them.

BACTERIOLOGICAL EXAMINATION.

Smears taken from the tumor tissue and stained by various methods have failed to reveal any structures suggesting living organisms, as has also examination with the dark-field microscope.

Cultures were made on all the usual laboratory media, both aerobically and anaerobically. No growth resulted, save on two tubes which showed evident contamination.

IMMUNITY.

Several experiments have been made in the attempt to demonstrate the occurrence of an immunity, natural or acquired, against the tumor. The results obtained up to the present hardly seem to justify definite conclusions, owing partly to the comparatively small number of animals tested, and partly to a lack of uniformity in the material used for reinoculation, as shown by the results in control fowls. The following is a summary of the findings.

In the 3d generation B there were inoculated three Plymouth Rock hens, three black Minorca hens, three brown Leghorn hens, and six mongrel pigeons. No tumor growth took place in any of the pigeons, suggesting, for that species at least, the occurrence of a natural specific immunity, as is the case with animal tumors in general. Tumors developed, however, in both of the alien breeds of the same species as the original host. A difference of breed does not, then, constitute a barrier to the transplantation of Chicken Tumor VII. In this respect the tumor contrasts sharply with Chicken Tumor I (Rous[6]), which in its earlier generations could be transplanted only to fowls of close blood relationship to the original host.

Reinoculations have been made in several series of animals, including fowls which remained negative from the first inoculation, fowls bearing growing or retrogressing tumors, and fowls in which the growth had retrogressed completely. Reinoculation of fowls negative from the original implantation gave tumors only in one series of three fowls. These had been inoculated originally with tumor emulsion, which gave completely negative results. The other fowls of this class, twenty-two in all, still showed no growth after the second inoculation. Although the value of the result was lessened, for a part of this series, by the unsuitable nature of the material used for reinoculation, the findings suggest the occurrence of a strong natural immunity in certain individuals of the species.

[6] Rous, P., *loc cit.*

In the case of fowls bearing tumors, or in which tumors had retrogressed, reinoculation gave a very small percentage of tumors as compared with the general results in normal fowls. The interpretation of the findings is complicated by several factors, which are important, one being the general poor health of fowls which have been confined for some time in close quarters. The inference is apparently justified, however, that a certain degree of resistance develops in animals in which the tumor has grown for any considerable period. If this is so it offers one explanation for the general tendency to retrogression which Chicken Tumor VII exhibits.

DISCUSSION.

The osteochondrosarcoma described occurred as a unique instance among some thirty primary chicken tumors brought to this laboratory during the last twelve months. It appears to have arisen from the periosteum, all the layers participating in the growth. Injury must be thought of as a contributory cause, because of the tumor's position. Its symmetrical arrangement and histological structure at first suggested that it was congenital in origin, but no evidence confirmatory to this view has been obtained.

In its general characters Chicken Tumor VII is like certain mouse tumors, also osteochondrosarcomata, described by Haaland[7] and Murray.[8] Murray's tumor was successfully transplanted and retained its ability to produce cartilage and bone. From his description it appears that these tissues were laid down in a spindle-celled parenchyma, much as happens in the case of Chicken Tumor VII. It is interesting in this connection to remember that dogs are not infrequently the subject of mixed tumors of the mamma which contain an epithelial element, cartilage, and bone, and occasionally give metastases showing one or more of these tissues. The development and differentiation which takes place in Chicken Tumor VII is somewhat similar to that occurring in the normal growth of the chick embryo, but in other ways is notably different. The tumor cells in their early form are relatively simple looking con-

[7] Haaland, M., *Ztschr. f. Krebsforsch.*, 1907, v, 125.

[8] Murray, J. A., *Third Scientific Report of the Imperial Cancer Research Fund*, 1908, 69.

nective tissue cells, and they become changed into elements which, save for the fact that they are hypertrophic, are wanting in none of the morphological characters of normal cartilage cells. At the same time an intercellular substance is formed which shows the morphological features and the staining reactions of the ground substance of normal cartilage. But between the connective tissue stage and the cartilage stage is an intermediate one during which the cell produces a large amount of collagenous material. Such a stage is not prominent in the development of the normal cartilage of the bird.

In many instances of Chicken Tumor VII the cartilage at length undergoes degeneration and death, possibly from insufficient blood supply. In others, or in other parts of the mass the cartilage is changed to an osteoid tissue which later undergoes calcification and erosion by osteoclasts, with resulting formation of bony trabeculæ. The bone formation takes place, as a rule, only in connection with the marginal healthy cartilage, so that a peripheral bony zone may be found enclosing a soft degenerated center (figure 3).

The process of bone formation differs in its order from that of normal endochondral bone in the chick, as described by Lillie,[9] for in the production of normal bone the cartilage is first calcified and then eroded, while at the same time osteoid tissue is deposited on the trabeculæ by osteoblasts. Whether in Chicken Tumor VII the bone marrow develops from neoplastic tissue or by an ingrowth of tissue elements from the host is unsettled, but the former process is suggested by the findings as an origin for many of the marrow elements, as is also the possibility of even certain of the blood cells having arisen from tumor tissue. Both hypotheses, of course, require more extensive confirmation.

SUMMARY.

An osteochondrosarcoma of the common fowl, designated in this laboratory as Chicken Tumor VII, has been successfully transplanted to seven successive series of hosts. The original growth contained bone and cartilage, was attached to the sternal keel of an otherwise healthy chicken, and appeared to have arisen from this

[9] Lillie, F. R., The Development of the Chick, New York, 1908, 409.

structure. In the growths derived from its transplantation cartilage is regularly laid down, followed later by bone in case the host lives long enough. The prechondral tissue consists of spindle-shaped or multipolar cells of the fibroblast type. The histological character and the behavior of this prechondral tissue show that it is sarcomatous, a fact further proven by one recent case in which the tumor has metastasized. The secondary growth in this instance consisted of prechondral tissue in which a cartilaginous change was taking place.

The tumor could not be transferred to pigeons, the one foreign species tested, but grew readily in chickens of two alien breeds. Reinoculation experiments suggest the occurrence of a natural, individual immunity, and of a certain degree of acquired resistance. In one fowl visceral growths developed following an intravenous injection of tumor emulsion, although whether they were due to this cause or were secondary to the large implantation growths in the muscles is uncertain. Recently the tumor has been transmitted by means of the filtrate from a Berkefeld filter.

EXPLANATION OF PLATES.

PLATE 79.

FIG. 1. Original tumor with a part of the sternal keel, viewed from the right side. The skin has been stripped away except over the crest of the keel. About three fourths natural size.

FIG. 2. Section through the center of the tumor and across the keel of the sternum. The keel lies vertically in the midst of the growth but is lost in a mass of spongy bone. A wedge-shaped piece of tissue has been cut from one side of the tumor. The dark areas deep in the growth are areas of spongy bone. *n* = the notch left by excision of the tissue; *k-k* = the position of the keel; *st* = the sternum at the base of the keel. About natural size.

PLATE 80.

FIG. 3. Cross section of a bony tumor resulting from transplantation. The growth was eighty-five days old and had been stationary for some weeks before its removal. In this instance a broad zone of spongy bony tissue surrounds a degenerated center. The growth is well encapsulated. About natural size.

FIG. 4. Original tumor. Microscopic preparations showing the disappearance of the sternal keel amid the growth's bony trabeculæ. At the upper left hand corner of the photograph is a dark mass of cartilage, which is separated from the bone by a layer of osteoid tissue nearly free from stain. *k-k* = lamellæ of the keel. Stained with hematoxylin.

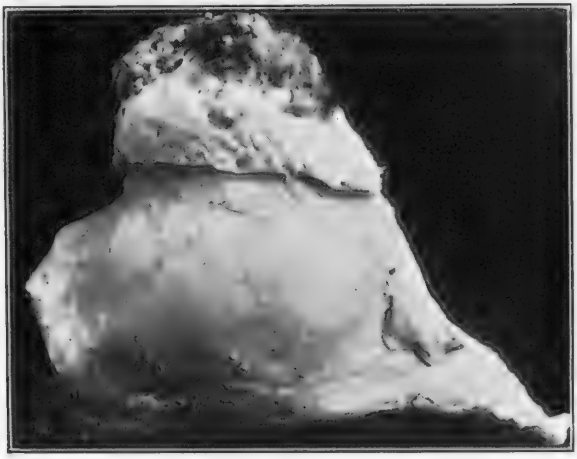


FIG. 1.

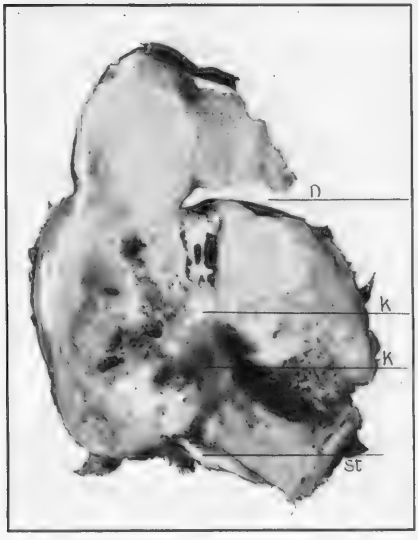


FIG. 2.

(Tytler: A Transplantable New Growth of the Fowl.)

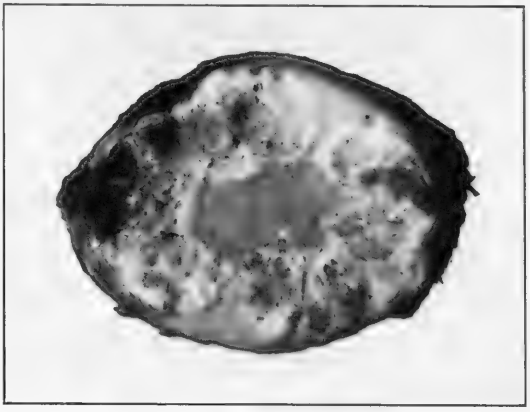


FIG. 3.

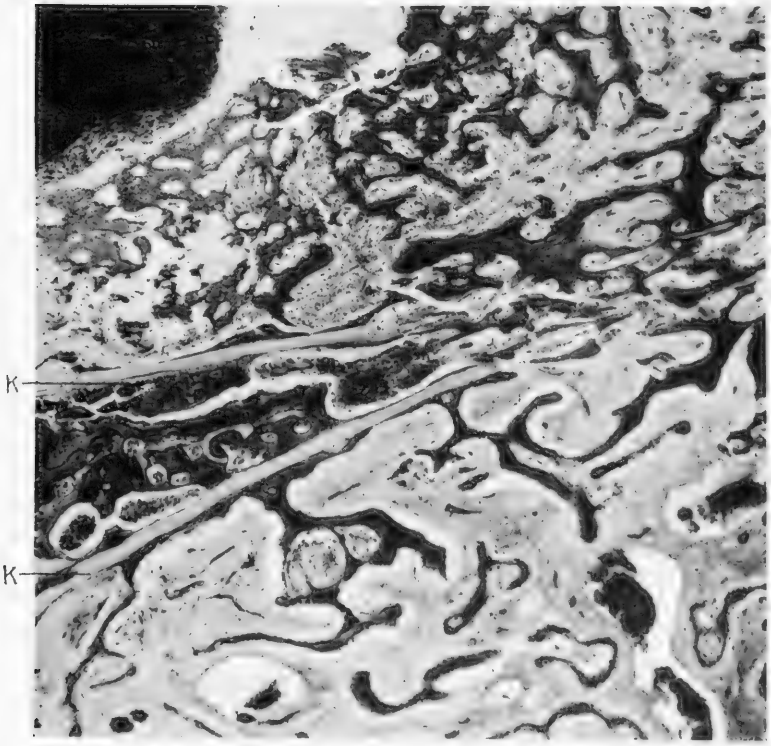


FIG. 4.

(Tytler: A Transplantable New Growth of the Fowl.)

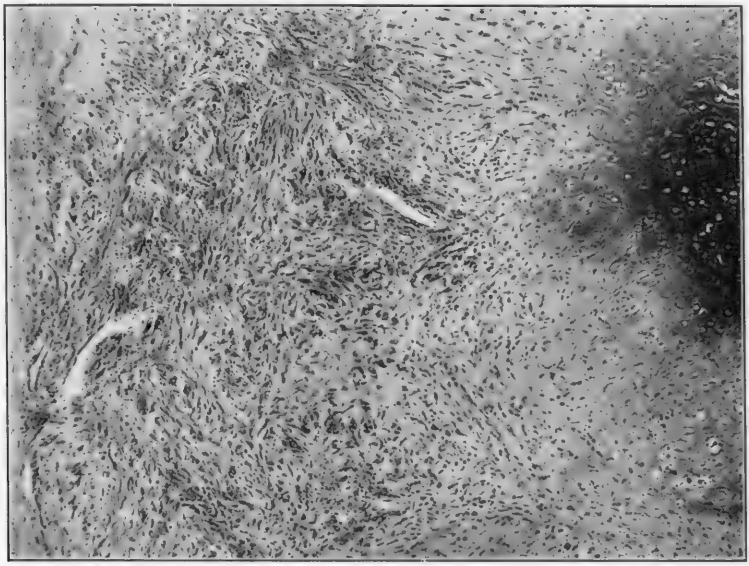


FIG. 5.

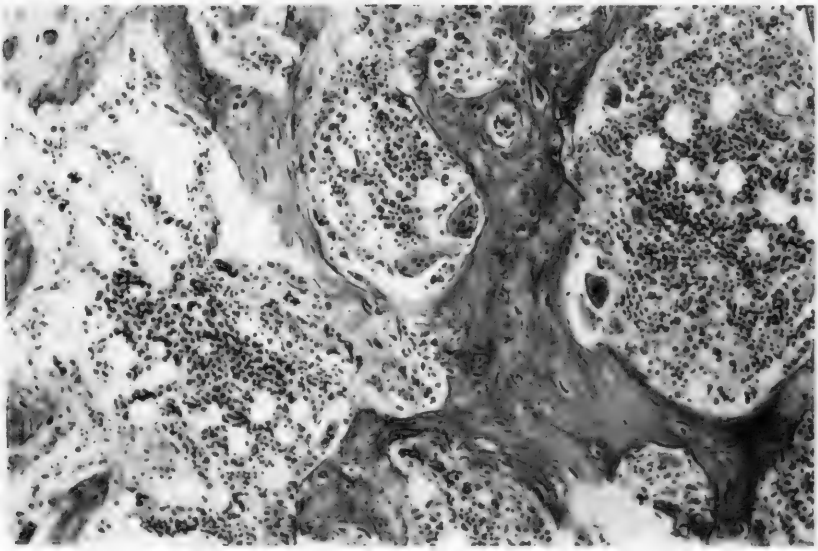


FIG. 6.

(Tytler: A Transplantable New Growth of the Fowl.

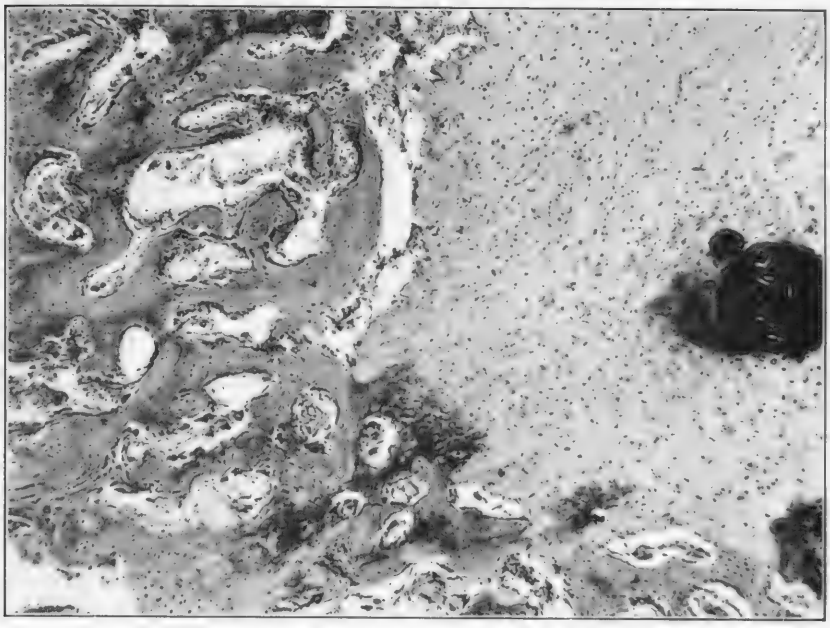


FIG. 7.

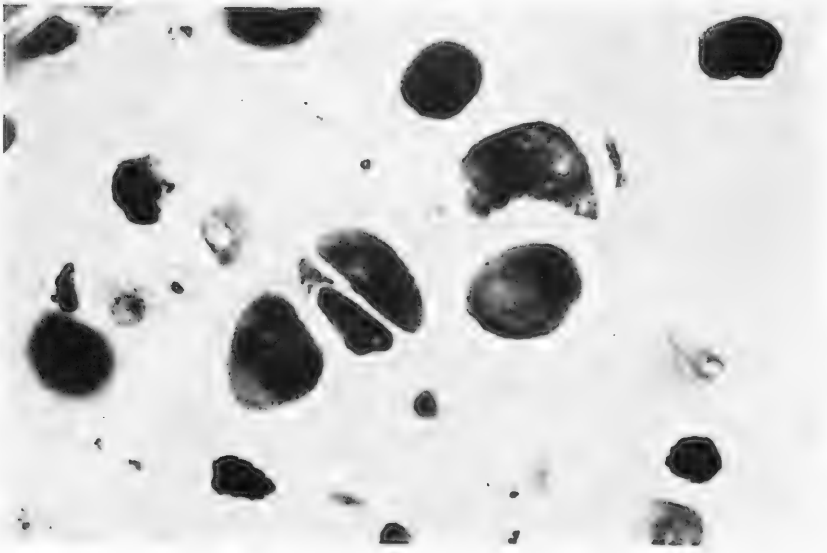


FIG. 8.

(Tytler: A Transplantable New Growth of the Fowl.)

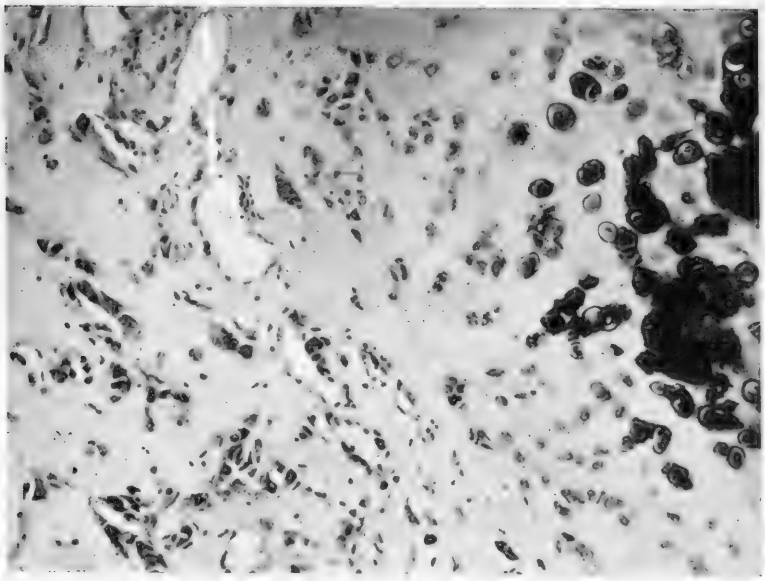


FIG. 9.

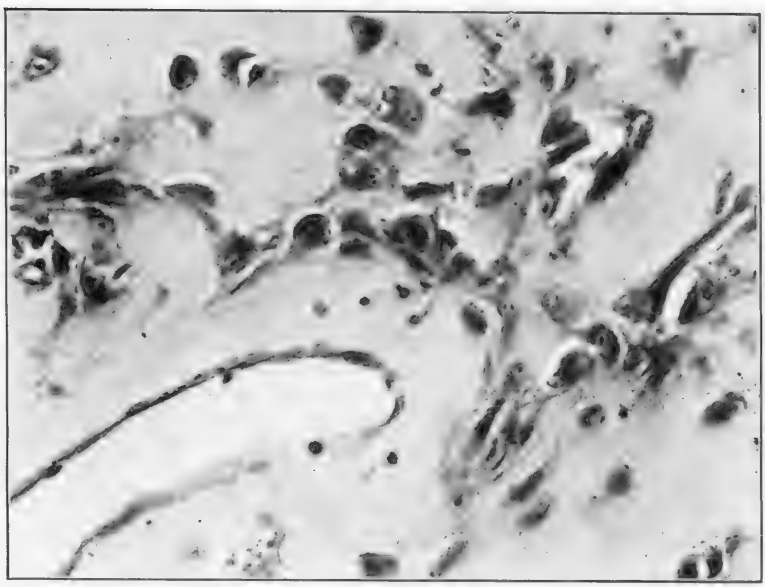


FIG. 10.

PLATE 81.

FIG. 5. Transplantation tumor (fowl 822, 1st generation). Duration of growth, thirty-five days. Microscopic preparation showing prechondral tissue and its transformation into cartilage. Stained with eosin and methylene-blue.

FIG. 6. Transplantation tumor (fowl 808, 1st generation), showing bony trabeculæ, osteoclasts, and marrow with myelocytes. Stained with hematoxylin.

PLATE 82.

FIG. 7. Original tumor. At the right of the picture there is cartilage, next to this osteoid tissue (nearly unstained), and to the left calcification of the osteoid tissue with marrow spaces advancing into the latter. Stained with hematoxylin.

FIG. 8. Hypertrophic cells of hyaline cartilage from a transplantation tumor (fowl 806, 1st generation). One cell has three nuclei. Stained with Mallory's chloride of iron hematoxylin.

PLATE 83.

FIG. 9. From a transplantation tumor (fowl 815, 1st generation), showing transformation of collagenous connective tissue to cartilage. Note the large blood sinuses. Stained with hematoxylin.

FIG. 10. Another portion of the same growth, more highly magnified, showing collagenous neoplastic tissue (prechondral tissue) with cells resembling fibroblasts. Part of a blood sinus walled by endothelial cells is also shown. Stained with Mallory's chloride of iron hematoxylin.

TRANSPLANTABILITY OF TISSUES TO THE EMBRYO OF FOREIGN SPECIES.

Its Bearing on Questions of Tissue Specificity and Tumor Immunity.*

By JAMES B. MURPHY, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York.*)

Plates 84 to 89.

Gardeners and arboriculturists have known for centuries that plants of one species could be grafted on to another without in any way affecting the essential characters of either. In more recent years it has been shown that a like condition exists in certain of the lower animals. For example, Joest[1] working with worms found it possible to bring about a permanent union between *Lumbricus rubellus* and *Allolobophora terrestris,* each of the segments retaining its individual characteristics. Born,[2] Harrison,[3] and Morgan[4] have extended these observations to tadpoles. They have noted that even when the grafted portion is small and absolutely dependent on the major component for its circulation, nervous system, etc., it retains the characters of the original species from which it was derived. Formerly it was considered possible to graft certain tissues from lower animals[5] to human beings, but a more careful consideration and repetition of the experiments which suggested this have shown them to be erroneous.

In recent years much has been added to our knowledge of the laws underlying tissue grafting in warm blooded animals through the study of the transplantable tumors. These in general are governed by the same principles as those that govern the transplantation of

* Received for publication, December 26, 1912.

[1] Joest, E., *Arch. f. Entwcklngsmechn. d. Organ.,* 1897, v, 419.

[2] Born, G., *Arch. f. Entwcklngsmechn. d. Organ.,* 1897, iv, 349.

[3] Harrison, R. G., *Arch. f. Entwcklngsmechn. d. Organ.,* 1898, vii, 430.

[4] Morgan, T. H., *Biol. Bull.,* 1899, i, 7.

[5] Allen, W., *Lancet,* 1884, ii, 875.

normal tissues. Not only do tissue grafts from warm blooded animals fail to survive when placed in a foreign species, but the barrier of tissue specificity also divides the different varieties of the same species. For example, certain tumors of the white mouse can not be successfully transplanted into grey, brown, or black mice of the same species, nor can certain tumors of the white rat be propagated in any other than white rats. Leo Loeb[6] has shown that the skin of a white guinea pig can be grafted on to the ear of a black guinea pig, but it eventually undergoes replacement with an inwandering of pigmented cells. A more recent and striking example of the strictness of the laws of specificity is that of a chicken sarcoma[7] which failed to grow in any other than blood-related animals during its first few transfers, later grew only in pure stock animals of the same variety, and would not grow in chickens of another variety until the malignancy had become greatly enhanced. This remarkable phenomenon of tissue specificity has been the subject of extensive investigation but its cause has not yet been determined. A general impression exists among experimenters that one of the immunity reactions is directed against the invasion of the foreign cells. The literature dealing with hemolysins and cytolysins need scarcely be gone into as the results reported offer no conclusive evidence. A theory of some interest, which explains the facts along other lines, is the athrepsia theory of Ehrlich,[8] which accounts for the failure of grafts in a foreign species by the lack of some specific food substance.

In the course of an investigation on the Rous chicken sarcoma in relation to chick embryo, it was observed that this tumor grew as well in the duck and pigeon embryo[9] (figure 1) as in that of the chicken, whereas it would not grow in the adults of these foreign species. This fact suggested to me the possibility that the chick embryo might serve as a suitable host for mammalian tissue as well. Inoculations of rat, mouse, and human tissue were made into the chick embryo and it was found that they grew as rapidly if not

[6] Loeb, L., *Arch. f. Entwcklngsmechn. d. Organ.*, 1898, vi, 1.
[7] Rous, P., *Jour. Exper. Med.*, 1911, xiii, 397.
[8] Ehrlich, P., *Arb. a. d. k. Inst. f. exper. Therap.*, 1906, No. 1, 84.
[9] Murphy, J. B., and Rous, P., *Jour. Exper. Med.*, 1912, xv, 119.

more so than in the adult of the native species[10] (figure 2). A typical experiment of this kind will be quoted.

Experiment 1.—A large rapidly growing Jensen sarcoma[11] of the rat was finely hashed and inoculated by means of a syringe and fine hypodermic needle into the outer membrane (fused chorion and allantois) of twelve chick embryos on the sixth day of incubation. On the eighteenth day of incubation the eggs were opened. Of the eight remaining alive, one showed no evidence of tumor; all the others showed a spherical mass projecting from the membrane at the point of inoculation. They varied in size from 0.1 to 1.6 cm. in their greatest diameter (figure 3). Histological examination showed these tumors to be made up of cells like those of the original tumor.

Bits of this tissue were inoculated into rats and there developed as a result tumors of the Jensen type.

Many experiments like the foregoing have been done. The growth of the Jensen tumor in the chick is always rapid. An accurate comparison of the rate of growth in the foreign embryo and the native adult would be of great interest, but this is rendered impossible on account of the nature of the injection site in the embryo. A certain proportion of the material injected into the egg escapes into the cavities and the amount clinging to the membrane and giving rise to the tumor varies on this account considerably. The results are sufficient, however, for the statement that the rate of growth is at least as rapid in the chick embryo as in the adult rat. It is of interest to note in this connection that while the normal temperature of the rat is 37.9°, the eggs were incubated at about 40° C.

DESCRIPTION OF RAT TUMORS IN THE CHICK EMBRYO.

For the work described in the present paper the Jensen sarcoma of the rat was used, a tumor widely known and the subject of much experimental work. Our strain gives a rapidly growing tumor in a high percentage of the animals inoculated. This tumor when introduced into the chick embryo grows readily where inoculated in the various membranes or in the body of the chick itself. For the general purposes of the experiment it has been found advantageous to use the outer membrane (fused allantois and chorion) for the reason that it lies just under the shell membrane and, therefore, can

[10] Murphy, J. B., *Jour. Am. Med. Assn.*, 1912, lix, 874.

[11] Jensen, C. O., *Ztschr. f. Krebsforsch.*, 1909, vii, 45.

be inoculated with a minimum amount of trauma. Furthermore, this membrane is the respiratory organ of the chick at this period and is rich in lymphatics and blood vessels. The inoculations were made between the fifth and seventh day of incubation and were allowed to grow till the eighteenth day.[12] Tumors resulting from such an inoculation of the Jensen sarcoma are found in the membrane as large globular masses lying in or suspended from the inner surface of the thin membrane by a broad pedicle. In the ten to twelve days of growth they sometimes attain the remarkable size of 2.1 cm. in diameter. The protruding masses are covered by a continuation of the chick membrane which gives them a smooth and glistening surface. Numerous dilated vessels are seen coursing through the membrane and penetrating the semitranslucent greyish tissue of the tumor itself. On section the nodules are found to be made up uniformly of this semitranslucent tissue, rarely with a small area of necrosis in the center but more frequently small areas of hemorrhage scattered here and there through the tissues.

Microscopically the cells making up these tumors resemble closely those of the same tumor in the rat; fairly large spindle cells with scant, deeply staining protoplasm and a large, clear vesicular nucleus with a nucleolus. They are so characteristic that there is no difficulty in distinguishing the tumor tissue from that of the chick even at the margin of the growth. The pattern of the tumor in the chick generally shows some variation from that in the rat (figures 4 and 5). In the latter the cells are seen in compact bundles coursing in various directions through the section, with a fair number of scattered, thin walled vessels. In the chick the arrangement of the cells is less compact, in some cases forming a loose network with clear spaces between, while in others the picture more closely approaches that seen in the native host. Whereas in the rat-grown tumors, mitotic figures are only occasionally seen, in those grown in the chick embryo they are found in almost every microscopic field, and as many as five mitotic figures have been seen in a single field of an oil immersion lens (figure 6). This finding is more striking when it is remembered that in the chick-grown tumors the number of cells

[12] Rous, P., and Murphy, J. B., *Jour. Am. Med. Assn.*, 1911, lvi, 741; Murphy, J. B., and Rous, P., *Jour. Exper. Med., loc. cit.*

per field is much less than in the rat-grown ones. The vessels are much more numerous in the tumors of the embryo, here occurring in two forms, either ingrowths as clusters from the chick membrane (figure 7) or as individuals scattered throughout the tumor (figure 8). Apart from the thin continuation of the chick membrane which covers the tumor and the ingrowth of vessels with their scant accompanying stroma, there is no histological evidence of reaction on the part of the embryo to the invasion of foreign tissue. Certainly there are none of the sort attributed to a defensive reaction under similar conditions in the adult host.

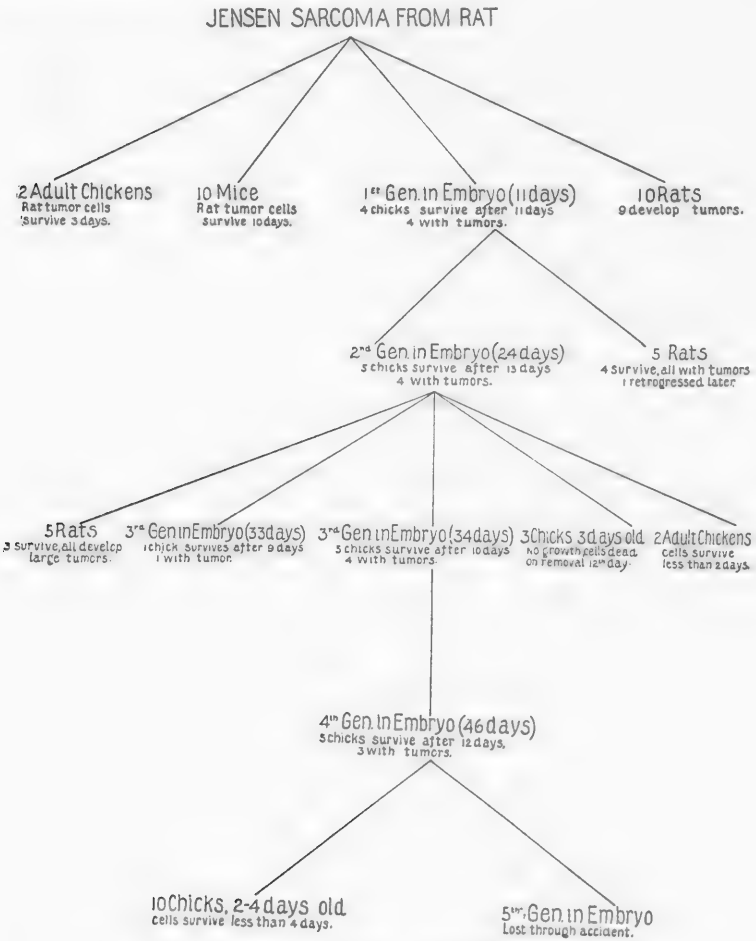
A microscopic study of the tumors occurring in the rat from inoculations with the chick-grown rat tumor shows them to be identical with those propagated in the usual way from rat to rat.

PROLONGED GROWTH OF RAT TISSUE IN CHICK EMBRYO.

The results of the experiments so far reported do not establish beyond question the utilization of the chick food material in the nourishment and growth of the rat cell, but they leave little doubt that this is the case. At the end of the twelfth day in the chick when the specific food carried with the graft from the native species must have been largely exhausted, there is no sign of lessened activity on the part of the tumor cells. Partly to settle this question beyond dispute, but more particularly to determine the changes, if any, that would be brought about in the rat cells by the prolonged growth in the embryos of a foreign species, the embryo-grown tumors were reinoculated into a second series of chick embryo. Not only did the tumors continue in rapid growth here, but they grew in turn when transplanted to a third or fourth series (figure 9). There seems to be no reason why by repeating transplantation growth cannot be prolonged indefinitely. Such an experiment with its controls will be quoted.

Experiment 2.—(Text-figure 1.) First generation. A rapidly growing Jensen rat sarcoma was finely hashed and injected into a series of seven day chick embryos. As control some of the same material was inoculated into the following animals: (1) ten rats, nine of which subsequently developed tumors; (2) ten mice, one of which was killed every forty-eight hours and whose tissues on microscopical examination showed survival of the rat cells till about the tenth day when they practically disappeared; (3) two adult

chickens, ten grafts in each, removed at twenty-four hour intervals, whose tissues showed survival of rat cells till the third day.[13] The eggs inoculated were opened on the eighteenth day of incubation, eleven days after the injection; four remained alive, all with large tumors.


JENSEN SARCOMA FROM RAT
2 Adult Chickens
Rat tumor cells survive 3 days.
10 Mice
Rat tumor cells survive 10 days.
1st Gen. in Embryo (11 days)
4 chicks survive after 11 days
4 with tumors.
10 Rats
9 develop tumors.
2nd Gen. in Embryo (24 days)
5 chicks survive after 13 days
4 with tumors.
5 Rats
4 Survive, all with tumors
1 retrogressed later
5 Rats
3 survive, all develop large tumors.
3rd Gen in Embryo (33 days)
1 chick survives after 9 days
1 with tumor.
3rd Gen in Embryo (34 days)
5 chicks survive after 10 days
4 with tumors.
3 Chicks 3 days old
No growth, cells dead on removal 12th day.
2 Adult Chickens
cells survive less than 2 days.
4th Gen. in Embryo (46 days)
5 chicks survive after 12 days,
3 with tumors.
10 Chicks, 2-4 days old
cells survive less than 4 days.
5th Gen. in Embryo
Lost through accident.

TEXT-FIG. 1. This represents experiment 2 in outline.

[13] The criteria used here for survival were preservation of cell outline and the retention of the basic staining properties of the nucleus.

Second generation. The tumors from the above series of embryo were hashed and inoculated as follows: (1) into five rats, four of which survived and all developed tumors; and (2) into a second lot of embryos on the sixth day of incubation. These were opened on the nineteenth day (thirteen days later), five remaining alive, four with tumors

Third generation. The material from the last series was hashed and inoculated into the following animals: (1) five rats, three of which survived, all developing tumors; (2) three young chickens, hatched less than three days before, all developing small nodules which on removal twelve days later were found to be made up of reactive tissue with no rat cells present; (3) two adult chickens, ten grafts each, which on removal at forty-eight hour intervals showed no rat cell surviving even after the first forty-eight hours; and (4) into a lot of eight day embryos, five of which survived till the eighteenth day, four with tumors. The total number of days in the embryo was thirty-four.

Fourth generation. All the material from the above series was used to inoculate another lot of six day embryos. On the eighteenth day five chicks remained alive, three with tumors. Total number of days in embryo, forty-six.

Fifth generation. The material from the above was inoculated into: (1) ten newly hatched chicks about three days old, grafts from which were removed at forty-eight hour intervals beginning with the fourth day. None of the grafts showed surviving rat cells. It was also inoculated into (2) a lot of seven day embryos but these were unfortunately lost through an accident to the heat-regulating mechanism of the incubator.

These rat tumors of the second and third generation in the chick were similar in practically every respect to those grown in one chick for twelve days. The cells showed no morphological changes, but the tissue was perhaps more compact than in the early tumors.

This experiment, showing as it does the ability of the rat cells to survive and grow actively in the chick embryo for at least forty-six days, makes it certain that the rat cells utilize successfully the food offered by the blood of the foreign species. The question of adaptation therefore comes up for consideration. During this growth in the chick, have the rat cells acquired a real adaptation to the strange host? Apparently not. Morphologically there are no changes in the cell (figure 10). The tumors transferred from chick embryo to chick embryo grow neither better nor worse in the later periods than in the first chick to which they were transplanted. The material when returned to the rat caused a rapidly growing sarcoma which is always of the same type as the original tumor.

A comparative study of the fate of rat cells taken directly from the rat and those grown for a period in the embryo, when implanted in an adult, has proved of interest. Some of these results are men-

tioned in experiment 2, and numerous other experiments have been carried out along the same line. As previously mentioned the cells taken directly from the rat and inoculated into the adult chick will survive about three days. The period for the newly hatched chick is about the same. When, however, the rat cells have been grown for a period in the chick embryo and are then placed in the adult chicken, no evidence of them can be found after twenty-four hours. Practically the same results are obtained when newly hatched chickens are used as hosts. Instead of an adaptation to the new conditions the rat cells appear to have become less resistant during their growth in the chick embryos. All these findings show that the tumor cells have retained their essential characters despite the strange environment. Whether a longer dependence on a foreign species embryo would affect an adaptation remains to be seen, but certainly the present results offer little encouragement in this direction.

The conditions that make a chicken an unsuitable host for the growth of a foreign species cell seem, according to these experiments, to develop during the last two days of shell life, for it is certainly not present before that time and is present at the time of hatching. An attempt is being made now to establish the nature of this reaction and the exact time of its development.

GROWTH OF OTHER TISSUES IN THE CHICK EMBRYO.

Besides the rat sarcoma a variety of other tissues have been grown in the chick embryo for longer or shorter periods. Highly organized tissues of the chick itself, which fail to grow or grow poorly on transplantation to the adult, will grow in the embryo. Such tissues as kidney (figure 11), testicle, ovary, bone, cartilage, etc., have been grown for a period of from seven to ten days, but as yet no attempt has been made to carry them farther. Embryomata formed by inoculation of hashed chick embryo can be carried through several generations. They resemble the embryomata seen in the adult. After several transplantations of the embryomata the epithelial elements are less evident, the bone, cartilage, and connective tissue predominating.

Several other tissues of foreign species were grown with success,

among them the Ehrlich sarcoma and chondroma of the mouse, embryomata of the rat and mouse, a mammary carcinoma of the mouse (figure 12), and the Flexner-Jobling adenocarcinoma of the rat. Attempts with human tissue met with only moderate success. due perhaps to the time that elapsed between removal from the body and the introduction into the eggs. However, with this exception the tissue grew actively, with numerous mitotic figures and a copious blood supply from the embryo.

DISCUSSION.

The question of an adaptation of the rat cells to a new host is naturally of great interest. The evidence of change in the tissue is slight, if present at all. Instead of becoming capable of a longer survival in the adult or even newly born individual of the foreign species after the long dependence on its embryo, the rat cells have become more susceptible to the unfavorable conditions existing in these hosts. As mentioned above, this retention of characteristics has an analogy in the plants and lower animal forms. The branches of one species of tree may be grafted into the trunk of another, but though it depends upon the sap supplied by the roots of a foreign species, it retains all its own characteristics. In the lower animals where it is easy to graft parts of closely related species, it is observed that where small pieces of one species can be grafted on to another, the minor component will retain its characteristics. When a part of this minor component is removed, the regeneration that takes place will be like the minor component, although dependent on the major component of a foreign species for its nervous system, alimentary tract, and vascular system.

The fact that rat cells will grow in the chick embryo almost until the time of hatching, and when transferred to second, third, and fourth lots are found at the end of forty-six days of continuous growth in the foreign species embryo to be growing as actively as the same tissue in the rat, leaves no possibility of doubt that the avian food is just as suitable as the native food for the maintenance and growth of the tissue. The inability of the embryo to elaborate a defensive substance against this invasion is not surprising when it is remembered that in very young animals certain of the immunity

substances, as hemolysins, can only be developed to a very slight extent or not at all.[14]

With the evidence at hand from these experiments, it would seem that the athrepsia theory of Ehrlich does not apply to this special case. We have evidence of the ability of the rat cells to utilize nutritive substances from the embryo of a foreign species. It might be suggested that the embryo offers a food of less specific composition and therefore utilizable by a foreign species cell. The evidence presented by the experiments in adults would favor another interpretation. If the temporary survival and growth of the cells when transferred to an adult of a foreign species is to be accounted for by the specific food carried with the graft from the native animal, we should expect the tissues to live an equal length of time whether in a closely related or more distantly related species. But such is not the case. I have shown here that while the rat cells can live for from nine to eleven days in the mouse, they die off in the adult chicken by the third day. It would seem much more likely that there is a defensive mechanism whose strength and rapidity of reaction depend upon the degree of relationship, being more prompt and violent the more foreign the tissue introduced. We have at least one example in immunity reactions of the fine gradation between species, namely, in the precipitin phenomena.[15] Here a precipitin developed for the sera of one animal will give a heavy precipitate with all sera from animals of the same species and a progressively fainter reaction with sera of other species, depending on the distance of relationship.

The type of reaction that prevents the growth of a tissue in a foreign species adult is as yet a matter of speculation. That it is a property which is developed rather than one naturally present in the adult is strongly suggested by the experiments of Russell.[16] He found that the cells of a mouse survived and multiplied in a normal rat for more than nine days, while in a rat previously immunized the

[14] Famulener, L. W., *Collected Studies from the Research Laboratory, Department of Health, New York*, 1911, vi, 199.

[15] For the literature and a comprehensive review of the subject, see Nuttall, G. H., Blood Immunity and Blood Relationship, Cambridge, 1904.

[16] Russell, B. R. G., *Third Scientific Report of the Imperial Cancer Research Fund*, 1908, 341.

graft was rapidly disintegrated, all cell outlines being obliterated by the fourth day. Lambert and Hanes[17] have added the observation that rat and mouse tissues will grow almost as well in plasma from an alien as from the native species. They have further shown[18] that if the animal from which plasma is obtained is previously immunized with the living cells of the foreign species, the plasma will inhibit or actually prevent any such activity. It has been suggested that this may be due to a cytotoxin. We have little conclusive evidence either from these experiments of Lambert and Hanes or from mine as to the exact nature of this defensive mechanism. Little more can be said at present than that such a defense exists.

SUMMARY.

Inoculation of the Jensen rat sarcoma into the developing chick embryo gives a rapidly growing tumor at the site of inoculation, whether in the membranes or in the body of the chick itself. These tumors by transfer from embryo to embryo can be kept going for as long as forty-six days, and perhaps indefinitely in the foreign species. The rat cells show no morphological change even after a very long dependence. Their biological characters are also retained, as is shown by the fact that the cells when replanted in the rat, after a prolonged sojourn in the chick, will produce a rapidly growing sarcoma of the Jensen type. These rat tissues grown for long periods in the chick show no adaptation to the new species, being destroyed even more rapidly when placed in the adult chicken than cells taken directly from the rat. Morphologically the cells retain a close resemblance to those in the original tumor.

Other tissues grown in chick embryo are various embryonic cells from the chicken, mouse, and rat, the Ehrlich sarcoma and chondroma of the mouse, a mammary carcinoma of the mouse, the Flexner-Jobling adenocarcinoma of the rat, and a human sarcoma.

EXPLANATION OF PLATES.

PLATE 84.

FIG. 1. A chicken sarcoma growing in the yolk sac of a pigeon embryo (*A*), seven days after inoculation.

[17] Lambert, R. A., and Hanes, F. M., *Jour. Exper. Med.*, 1911, xiv, 129.

[18] Lambert, R. A., and Hanes, F. M., *loc. cit.*, p. 453.

FIG. 1.

FIG. 2.

(Murphy: Transplantation of Tissue to Embryo.)

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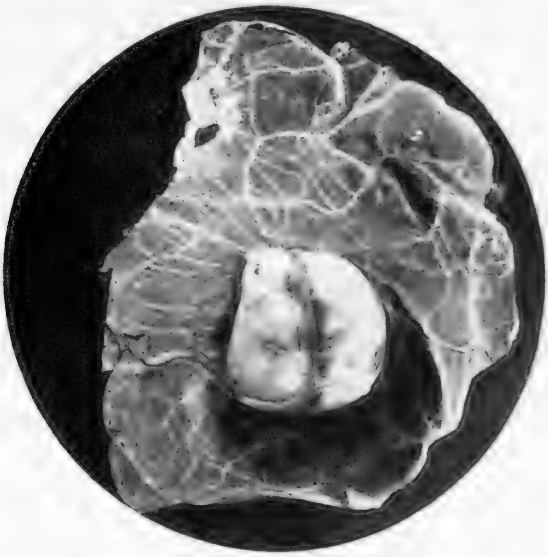

FIG. 3.

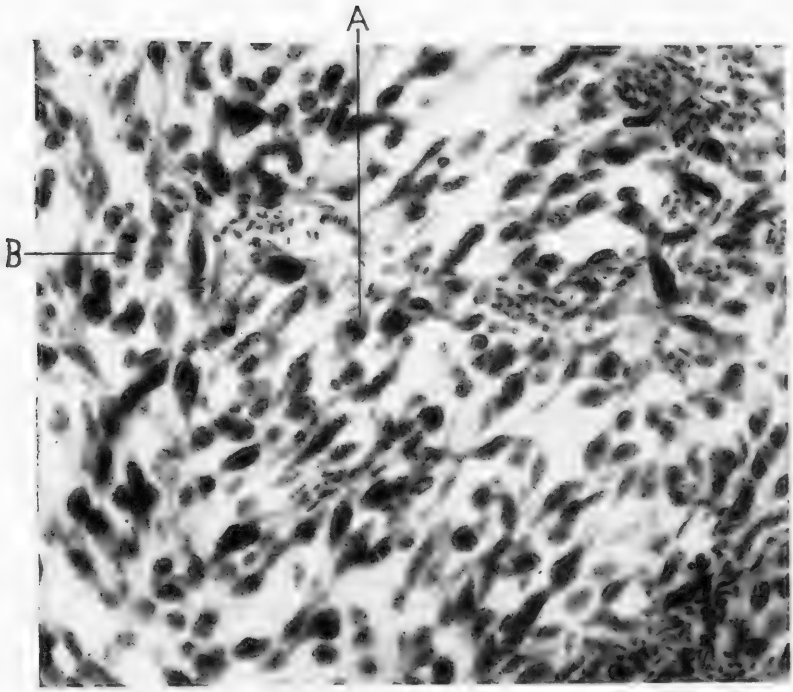

FIG. 4.

(Murphy : Transplantation of Tissue to Embryo.)

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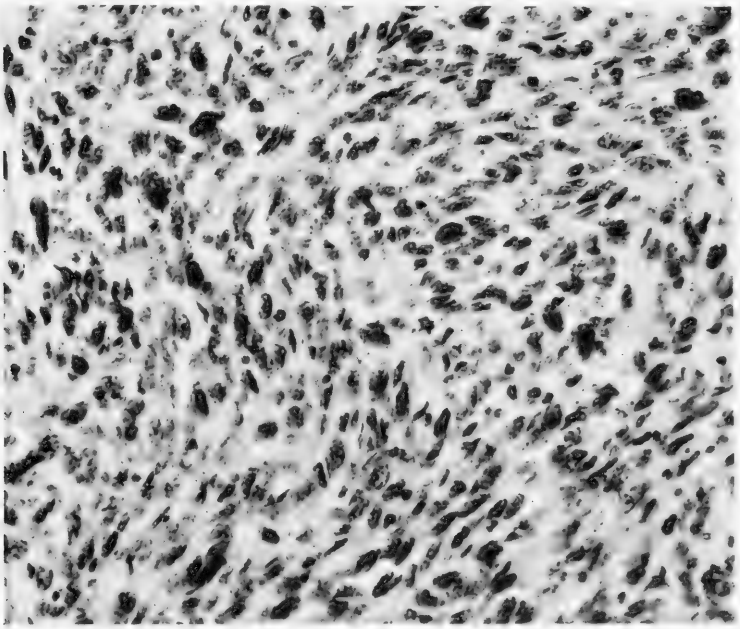

FIG. 5.

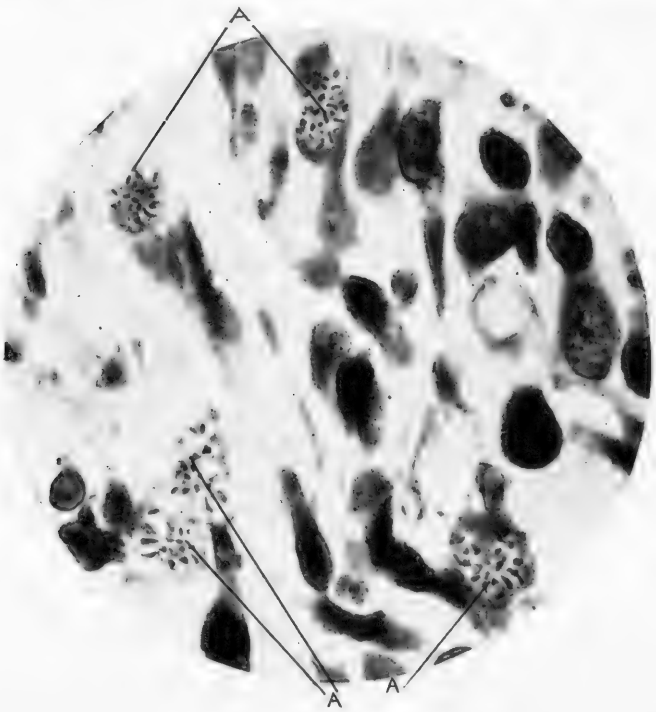

FIG. 6.

(Murphy: Transplantation of Tissue to Embryo.)

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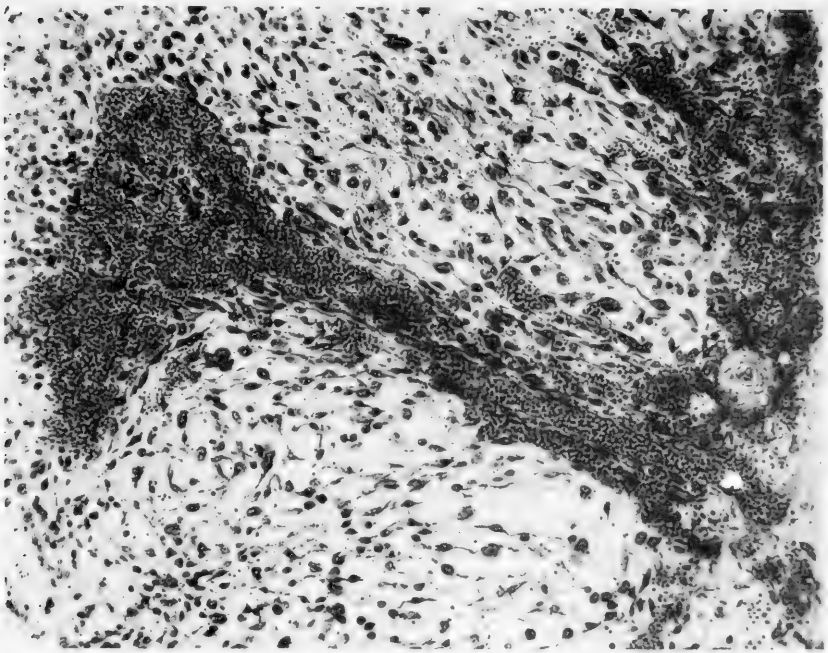

FIG. 7.

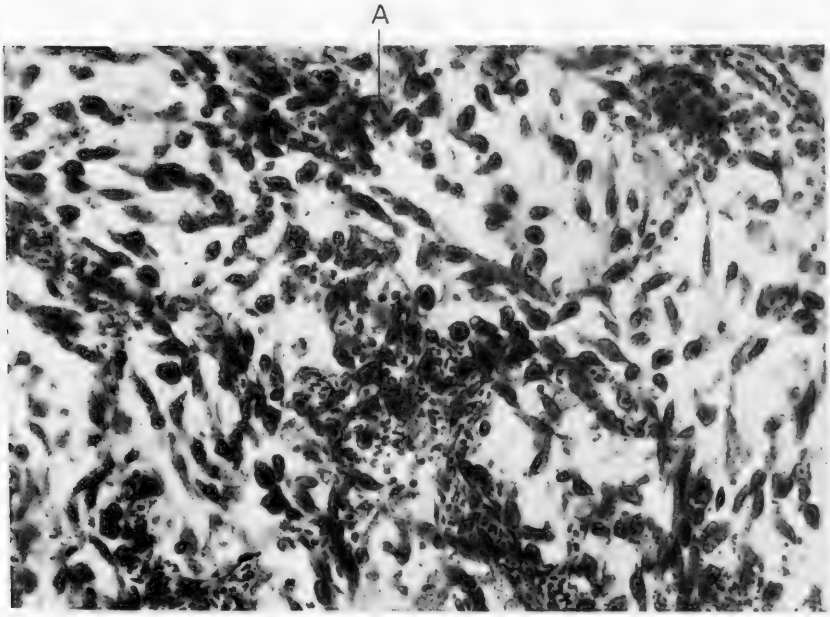

FIG. 8.

(Murphy: Transplantation of Tissue to Embryo.)

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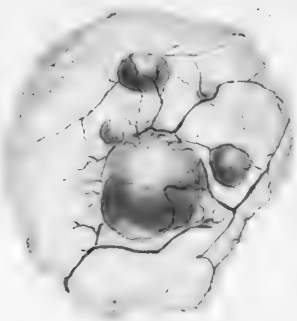

FIG. 9.

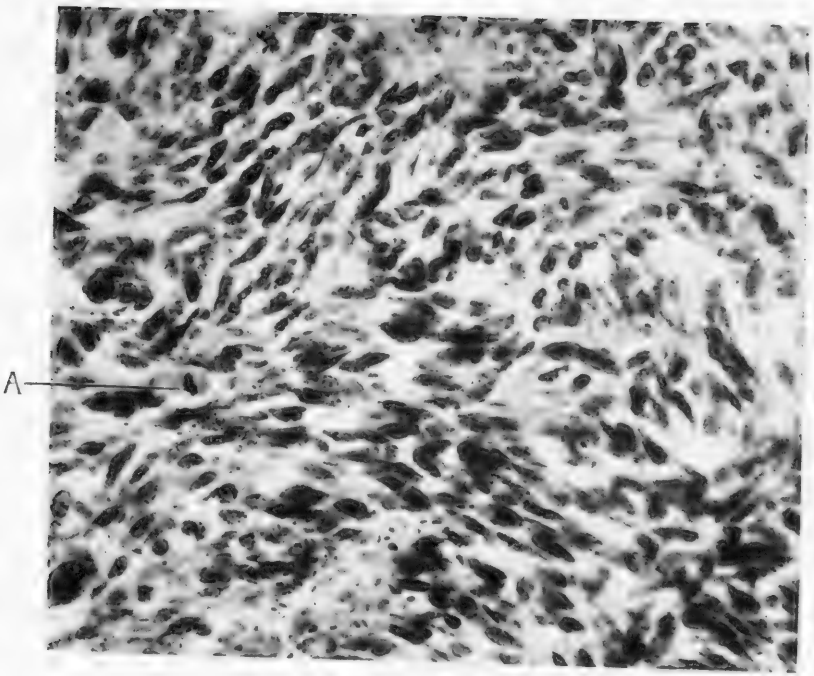

FIG. 10.

(Murphy: Transplantation of Tissue to Embryo.)

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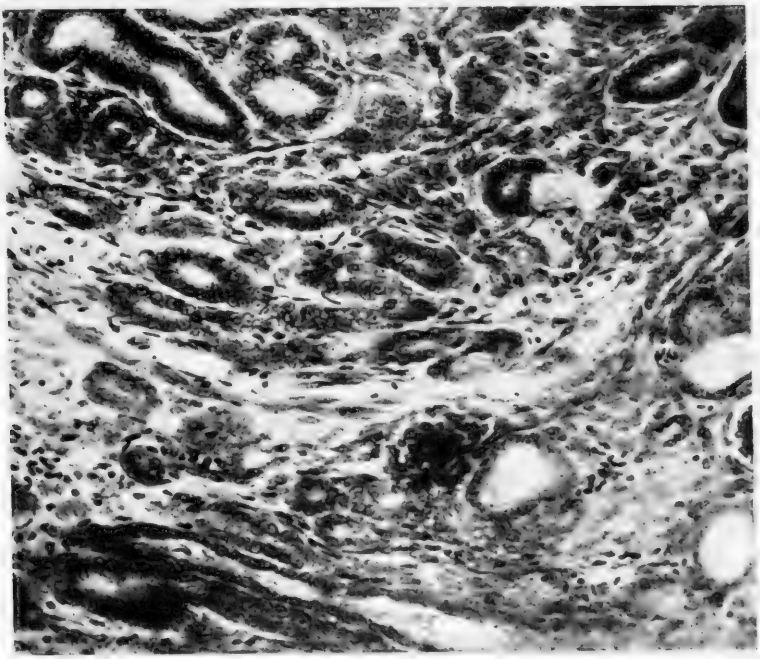

FIG. 11.

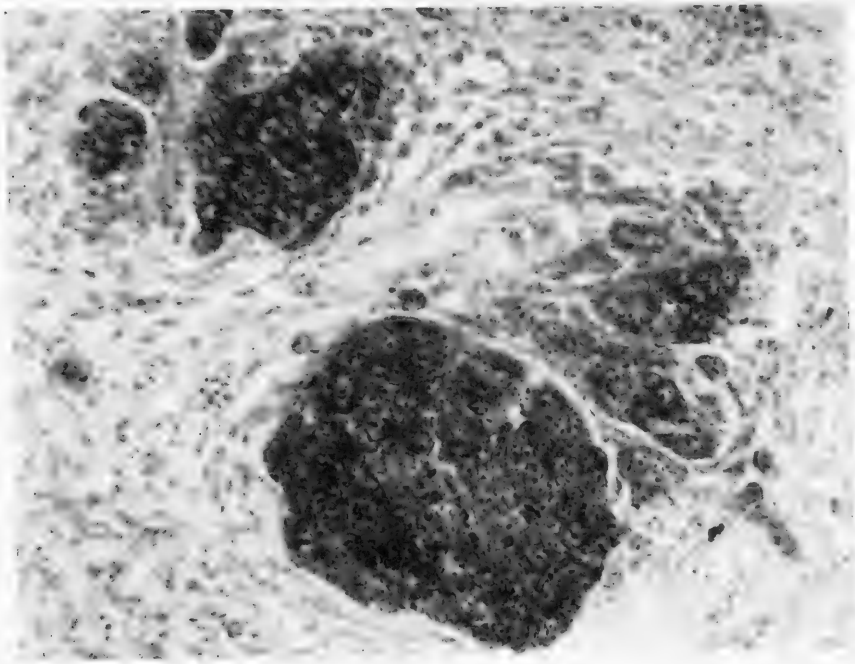

FIG. 12.

(Murphy: Transplantation of Tissue to Embryo.)

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FIG. 2. The Jensen rat sarcoma in a chick embryo. This animal was killed on the eighteenth day of incubation, seven days after inoculation. The tumor lay partly in the cranial cavity, extending through an opening in the skull and protruding outward.

PLATE 85.

FIG. 3. The Jensen rat sarcoma in the outer membrane of an eighteen day embryo, eleven days after inoculation. The tumor measured 1.7 by 1.5 cm.

FIG. 4. A section of rat sarcoma after twelve day's growth in a chick embryo. *A* and *B* = mitotic figures.

PLATE 86.

FIG. 5. The Jensen rat sarcoma as it appears growing in the rat.

FIG. 6. A rat sarcoma after eleven days in a chick embryo, showing five mitotic figures.

PLATE 87.

FIG. 7. A tuft of vessels from the chick membrane growing into a rat tumor which it supports. The membrane is on the right side.

FIG. 8. A second and more common type of vessel distribution in a rat tumor growing in chick embryos. The rat cells are seen clustered around the small scattered vessels. *A* = mitotic figures.

PLATE 88.

FIG. 9. A group of rat sarcomata in a chick membrane, second generation in the embryo, with a total of nineteen days of continuous growth in the foreign species. The central tumor measured 1.4 by 1.4 cm.

FIG. 10. A section of rat sarcoma after four generations in chick embryos (forty-six days). *A* = mitotic figures.

PLATE 89.

FIG. 11. Growing kidney tubules in the outer membrane of an eighteen day chick, resulting from the inoculation of embryonic kidney.

FIG. 12. A mammary carcinoma from a mouse growing in a chick embryo. The epithelial cells are seen in clusters.

FALSE TRANSITIONS BETWEEN NORMAL AND CANCEROUS EPITHELIUM.*

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PLATES 90 TO 97.

The question as to whether cancer arises directly from the normal structures amid which it first appears has considerable theoretical importance and has been one of the most debated in pathology. Yet despite much recent investigation it has not been entirely decided. Some workers report that the apparent transitions between normal and cancerous epithelium so often found are mere appositions, not transitions in the true sense of the term. In this conclusion they find a new argument for the theories which involve an independent origin for cancer. Other workers again describe what appear to be most convincing transition stages; and certainly pictures showing such transitions are very familiar to the consulting pathologist. For the purpose of the present communication the literature scarcely need be reviewed. It is not our aim to dispute the existence of true transition stages, or, on the other hand, to affirm it, but to demonstrate that the histological appearances of transition can result from a secondary union of normal and cancerous epithelium, and probably do so result frequently.

Several years ago while examining into the fate of implanted mixtures of embryo and tumor, the author noted in several instances a direct union of carcinoma with the stratified squamous epithelium of the embryo.[1] This came about when the normal and cancerous epithelium, growing along a surface, met end to end. The finding suggested an experiment, as follows:

Experiment.—Eight normal rats and seven carrying the Flexner-Jobling adenocarcinoma were used. The tumor employed was the adenocarcinoma just

* Received for publication, January 10, 1913.

[1] Rous, P., *Jour. Am. Med. Assn.*, 1910, liv, 1939; *Jour. Exper. Med.*, 1911, xiii, 239.

mentioned, a growth which had its primary origin in the seminal vesicle. Under ether a disc of skin was cut from each side of the normal rats and on the exposed surfaces (which did not bleed) there were spread with the flat of a knife a few fragments of finely ground tumor tissue. The dressing was a flexible paraffin mixture which became fluid at 47° C. Of itself this mixture does not induce atypical epithelial proliferation. In order to rule out a possible damage to the tumor tissue from heat the dressing was put on the wound as a thin, solid sheet warmed to flexibility. It was kept in place with gauze and adhesive plaster.

In some instances a second dressing was made on the sixth day, in others the first one remained undisturbed until the rats were killed and examined at the ninth to eleventh day. In only one rat did any infection develop. In all the epithelium rapidly regenerated and in several it had covered the wound surface by the ninth day. On twelve of the sixteen disc-shaped wounds a tumor developed as one or more discrete, whitish nodules lying for the most part below the surface and sometimes extending beyond the area denuded. In some instances the thin edge of epithelium advancing to cover the wound appeared intimately attached to the pale, exposed tumor. The specimens were cut into blocks vertical to the surface and serial sections were made of them.

In the case of the rats which already carried the Flexner-Jobling tumor at the time of operation the tumor tissue was exposed by slicing off the skin over it and a portion of the capsule. Infection was frequent and the exposed tumor usually became necrotic. In only two instances did the conditions appear favorable to a junction of the normal and carcinomatous epithelium. In one of these cases such a junction was found microscopically.

The results of the experiment are best shown by the photographs (figures 1 to 8). Even macroscopically there often appeared to be some connection between the thin film of regenerating epithelium and the exposed carcinoma. Microscopically a direct union of the two was not only frequent, but it many times took the form of a gradual histological transition between the stratified, squamous epithelium and the adenocarcinoma. That these are actual transitions involving a malignant transformation of the regenerating epithelium is scarcely possible. Previous knowledge is entirely against it; and, furthermore, the transition is always a transition to the peculiar type of adenocarcinoma that was inoculated,—a growth with cells much larger than those of the resting or regenerating squamous epithelium, containing a large and more deeply staining nucleus. The adenocarcinoma, when propagated alone, has shown no tendency to the squamous form.

The transition pictures are found under two conditions. The regenerating epidermis may not yet have extended over the carcinomatous surface and has united at its advancing edge with the

neoplastic epithelium (figures 1, 2, and 4); or the epidermis has more or less completely covered the tumor, and prolongations from it into the deeper tissue connect directly with strands or acini of the carcinoma (figures 3 and 5). Probably in the latter instance there was originally an end-to-end meeting on or near the surface, but the regenerating epidermis spreading beyond this junction has placed it underneath.

In addition to transition stages there have been found many meeting points of skin and cancer without any union of the two (figure 4), and many in which a union occurred but a definite line of demarcation between the two remained (figures 6 and 7). Occasionally individual carcinoma cells appear to have been caught up in the regenerating epithelium and, proliferating subsequently, to have extended out from it (figure 8). This finding is of interest in connection with the doctrine of "embryonic rests." If such rests exist in normal epithelium, their proliferation might very well result in such pictures, or in transition pictures that would completely conceal the real origin of the tumor.

There seems to be no doubt that, if a squamous-celled carcinoma had been available for the experiment, even more deceptive transition pictures than the ones figured would have been obtained. Each small ulceration of an epithelioma of man gives opportunities similar to those in the experiment for a secondary union of the cancer with the normal skin and thus for the development of transition pictures. The findings detailed in the present paper should make for greater caution in the interpretation of the histological appearances of transition between normal and cancerous epithelium.

EXPLANATION OF PLATES.

PLATE 90.

FIG. 1. End-to-end union of the regenerating epidermis and the adenocarcinoma. *sk* = epidermis; *c* = carcinoma; *sq* = epidermal pearl.

At *T* there is a histological transition between the carcinoma and the epidermis.

PLATE 91.

FIG. 2. To be viewed in connection with figure 3. End-to-end union of the regenerating epidermis and the adenocarcinoma.

FIG. 1.

(Rous: Transitions between Epithelium.)

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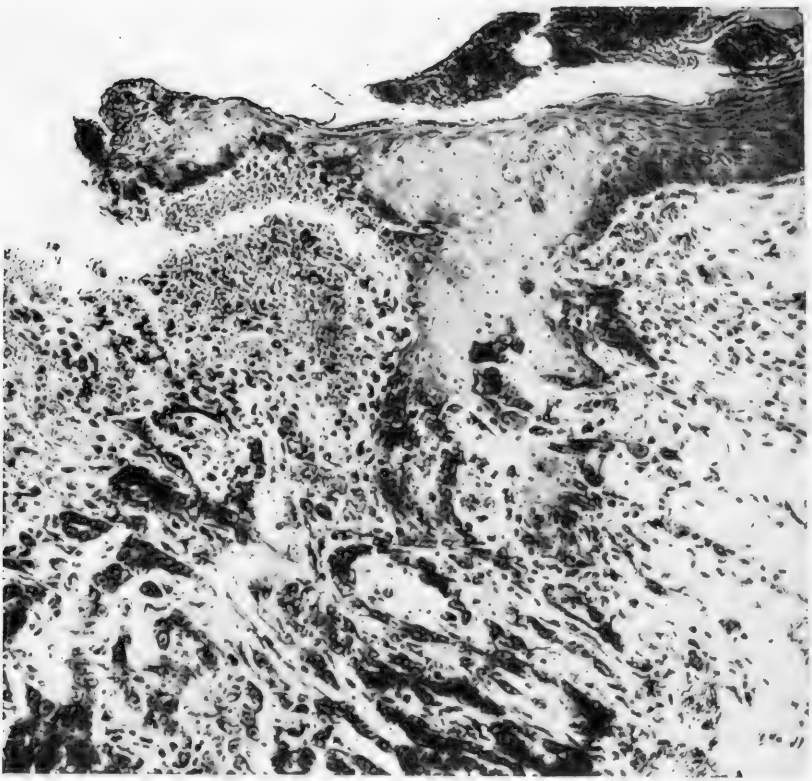

FIG. 2.

(Rous: Transitions between Epithelium.)

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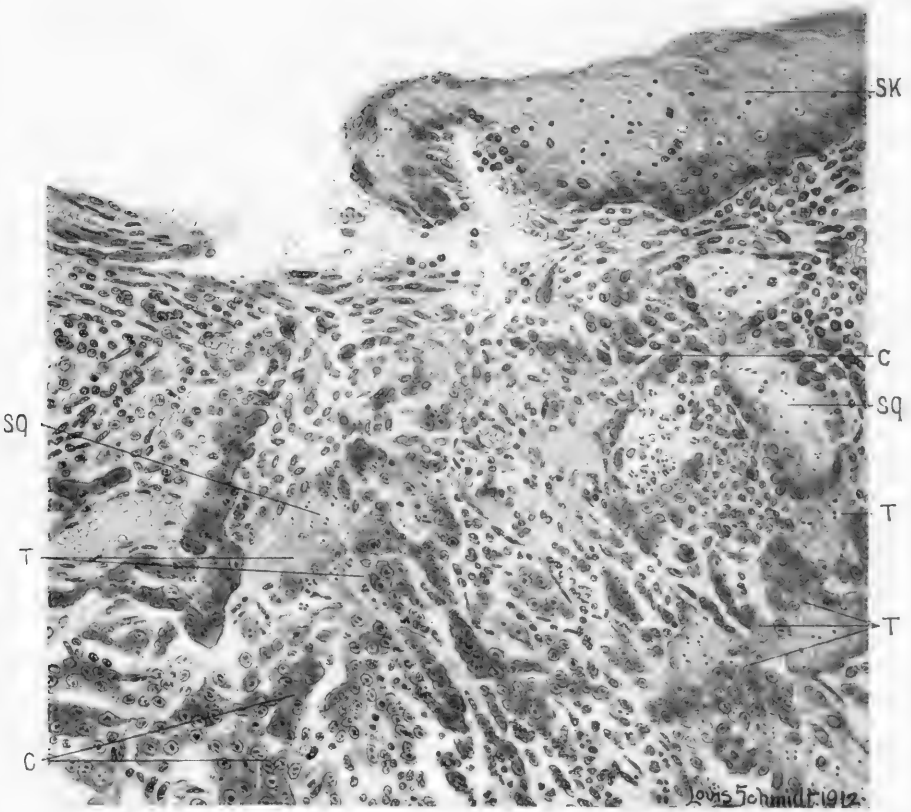

FIG. 3.

(Rous: Transitions between Epithelium.

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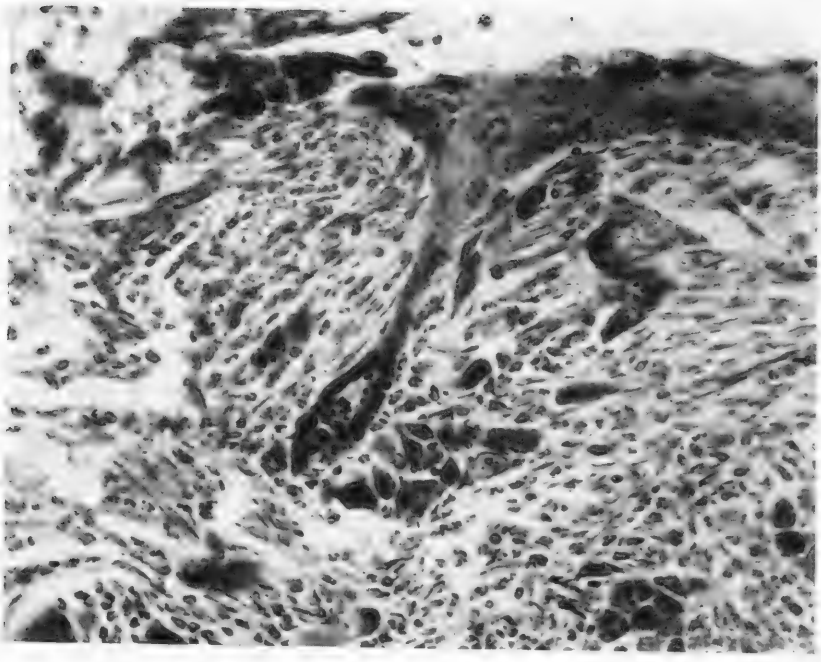

FIG. 4.

(Rous: Transitions between Epithelium.)

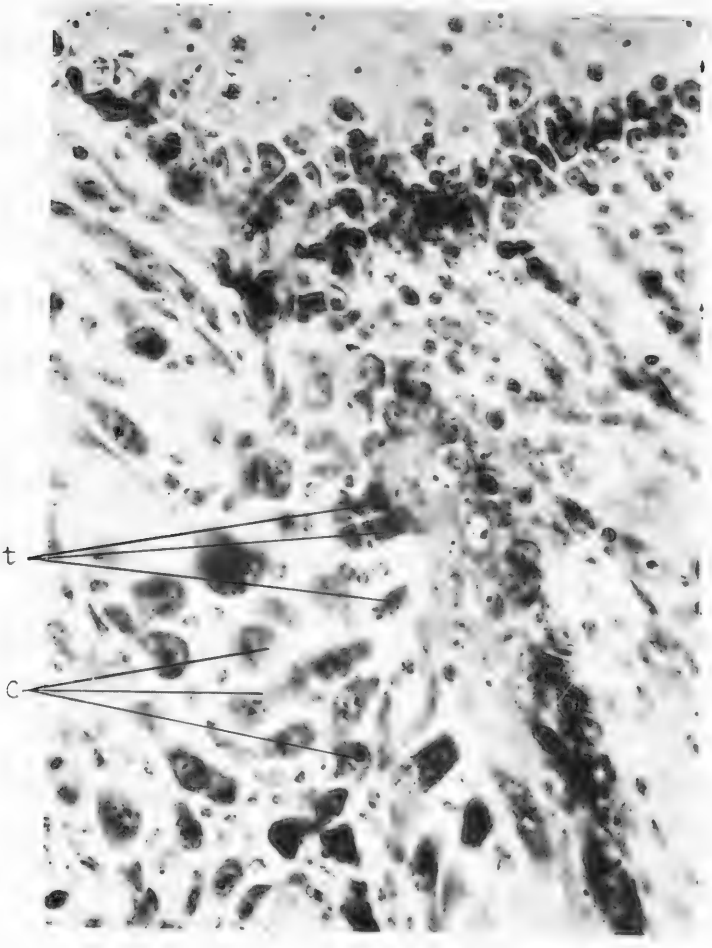

FIG. 5.

(Rous: Transitions between Epithelium.)

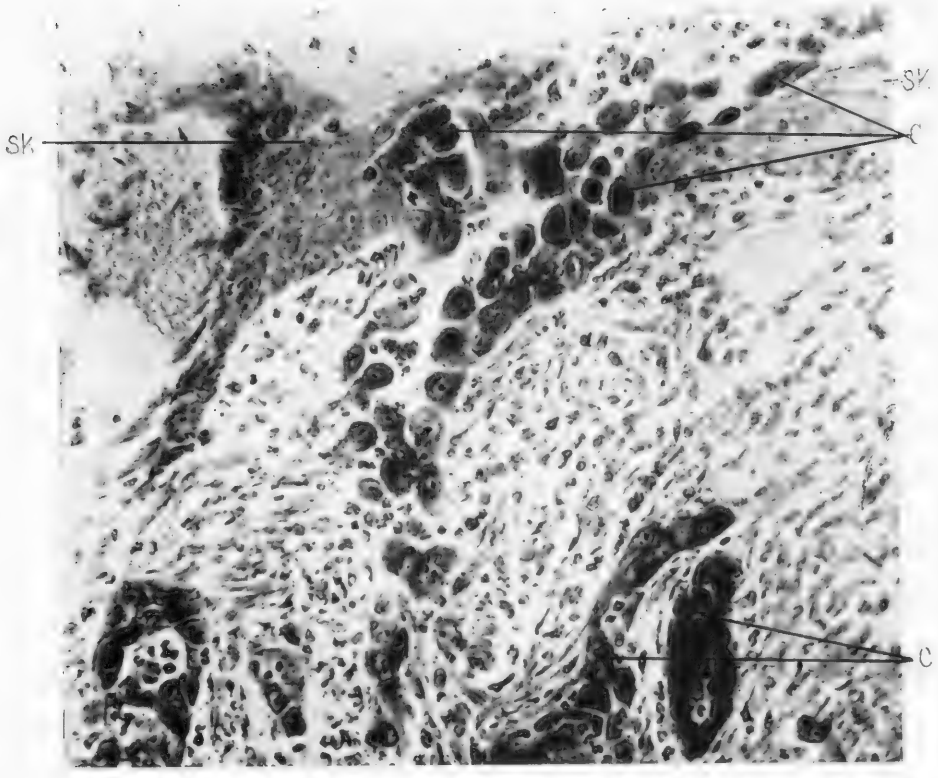

FIG. 6.

(Rous: Transitions between Epithelium.)

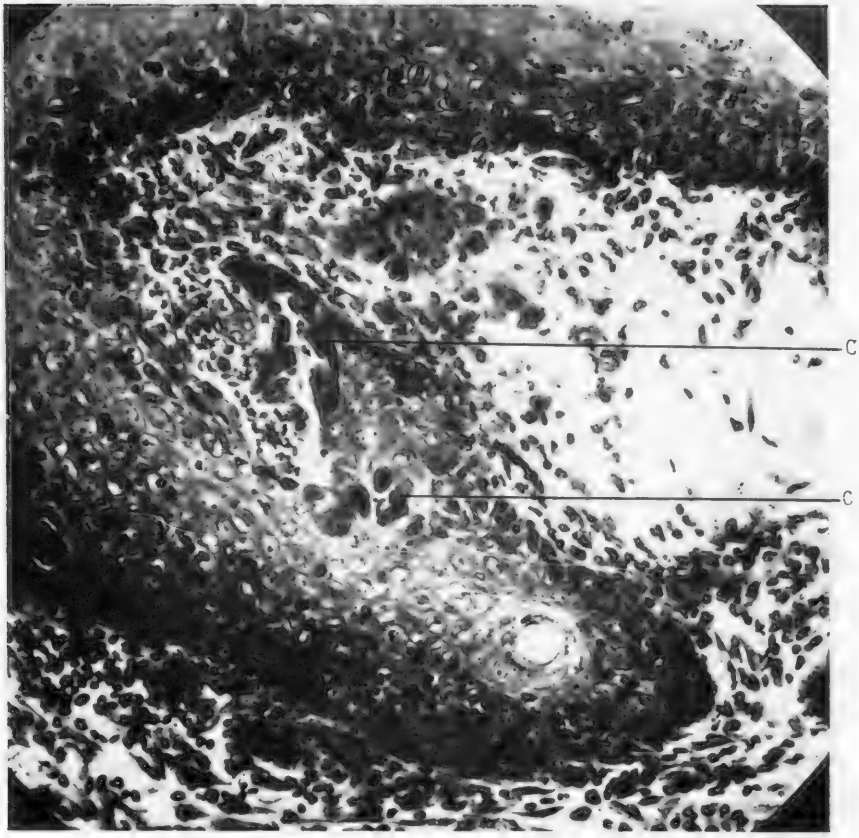

FIG. 7.

(Rous: Transitions between Epithelium.)

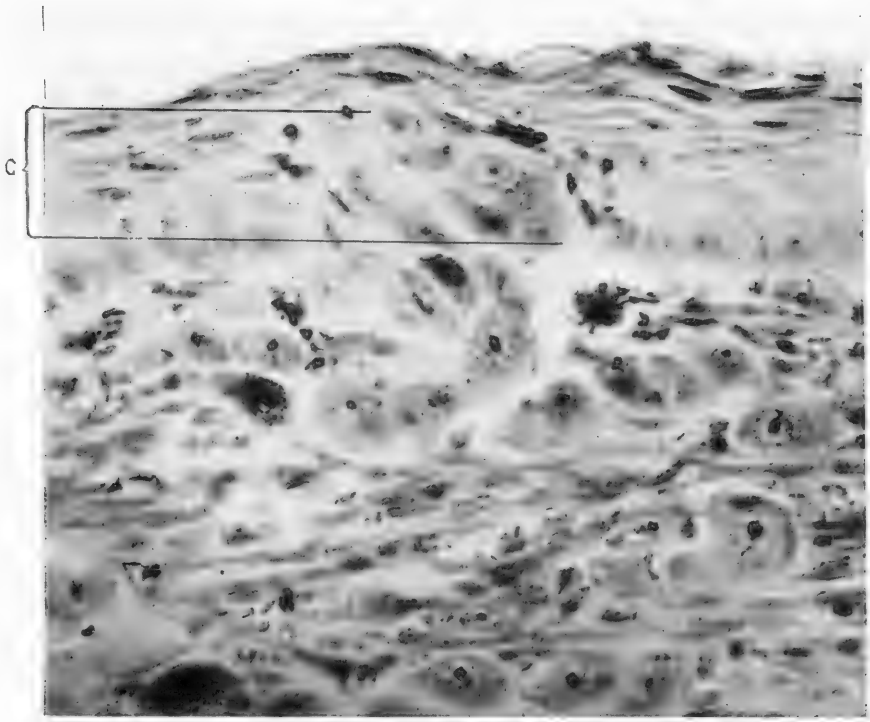

FIG. 8.

(Rous: Transitions between Epithelium.)

PLATE 92.

FIG. 3. Another section of the specimen shown in the preceding figure. The connection between the squamous epithelium here found deep in the tissue and the surface epithelium is made plain by figure 2. *sk* = regenerating epidermis; *c* = carcinoma; *sq* = squamous epithelium of epidermal prolongations into the deeper tissue; *T* = union between the carcinoma and the squamous epithelium, with transition forms.

PLATE 93.

FIG. 4. Union of the carcinoma and the basal layer of cells of the regenerating epidermis. The histological transition between the epidermal cells and those of the cancer acinus is well shown. At the surface of the wound there is a meeting between the epidermis and carcinoma but no union.

PLATE 94.

FIG. 5. Union of individual carcinoma cells with those of a downward prolongation of the regenerated epidermis. At the upper border of the figure is a portion of the surface layer of epidermis. Its connection with the prolongation at the center of the picture is not shown. *t* = tumor cells united with the prolongation; *c* = free tumor cells.

PLATE 95.

FIG. 6. Juxtaposition of carcinoma cells and those of the regenerating epithelium, without transition forms. *c* = carcinoma; *sk* = epidermis.

PLATE 96.

FIG. 7. Juxtaposition of carcinoma cells and those of a prolongation of the regenerating epithelium. No transition forms. *c* = carcinoma.

PLATE 97.

FIG. 8. An island of carcinoma cells (*c*) lying within the regenerated epidermis. Numerous carcinoma cells are scattered in the deeper tissue.